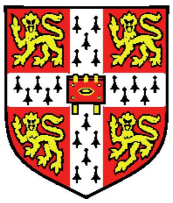
Estrogen Receptor Beta Modulates Prostate Carcinogenesis



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Doctor of Philosophy

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This work is dedicated to men with prostate cancer, for whom we don't yet have all the answers and for whom we must continue our efforts to better understand the disease.

Declaration of Originality

This dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration except as specified in the text. It is not substantially the same as any that I have submitted, or, is being concurrently submitted for a degree or diploma or other qualification at the University of Cambridge or any other University or similar institution. I further state that no substantial part of my dissertation has already been submitted, or, is being concurrently submitted for any such degree, diploma or other qualification at the University of Cambridge or any other University or similar institution. It does not exceed 60,000 words as specified by the Degree Committee.

Adam W. Nelson

26th June 2017

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Abstract

Prostate cancer (PC) is characterised by dependence upon androgen receptor (AR) as its driving oncogene. When organ-confined, radical treatment can be curative, however there is no cure for advanced, castration-resistant prostate cancer (CRPC). There is therefore a need to better understand the biology of PC, and how influencing AR can modify disease progression.

Estrogen is essential for prostate carcinogenesis with evidence from epidemiological, *in vitro*, human tissue and animal studies. Most suggests that estrogen receptor beta (ERβ) is tumour-suppressive, but trials of ERβ-selective agents have not improved clinical outcomes. ERβ has also been implicated as an oncogene, therefore its role remains unclear. Additional evidence suggests interplay between ERβ and AR, the mechanisms of which are uncertain. The study hypothesis 'ERβ is an important modulator of prostate carcinogenesis' was developed to establish whether targeting ERβ could affect PC progression.

Much of the confusion around ERβ stems from use of inadequately validated antibodies and cell line models. The first phase of this work was to test ERβ antibodies using an ERβ-inducible cell system. Eight ERβ antibodies were assessed by multiple techniques, showing that commonly used antibodies are either non-specific or only specific in one modality. Two reliable antibodies were identified.

Next, cell lines previously used to study ERβ were assessed using

validated antibodies and independent approaches. No ERβ expression was detected; an important finding that casts doubt on previously published ERβ biology. Subsequently, a PC cell line with inducible ERβ expression (LNCaP-ERβ) was developed and validated to enable controlled experiments on the effects of ERβ on proliferation, gene expression and ERβ/AR genomic cross-talk.

Phase three of this work focused on ERβ biology in PC and its relationship to AR. Interrogation of clinical datasets showed that greater ERβ expression associated with favourable prognosis. Gene expression data from men treated with androgen deprivation therapy revealed that AR represses ERβ. This was confirmed *in vitro*. The LNCaP-ERβ cell line was treated with androgen and/or ERβ-selective estrogen. Activated ERβ in the presence of androgen-stimulated AR inhibited cell proliferation and down-regulated androgen-dependent genes. Genome-wide mapping of ERβ binding sites reveals that ERβ antagonises AR through competition for shared DNA binding sites.

In conclusion, ERβ expression is down-regulated by AR during malignant transformation of prostate epithelium. We reveal an antagonistic relationship between ERβ and AR whereby sustaining or replacing ERβ may inhibit tumour growth through down-regulation of AR-target genes. In future, an ERβ-selective compound may be used to slow or abrogate PC progression.

Contents

List of Figures

List of Tables

Chapter 1

Introduction

1.1 Clinical aspects of Prostate Cancer

1.1.1 Epidemiology

Prostate cancer is the commonest, non-cutaneous cancer in men, affecting 21% of men in Europe. It is the second most common cause of male cancer-related death, accounting for 15% of deaths in developed countries [Heidenreich et al., 2011; Mottet et al., 2011]. In the UK, recent estimates suggest an annual incidence of 111 cases per 100,000 people, with an age-standardised mortality rate of 23 per 100,000 [Ferlay et al., 2013]. It is primarily a disease of elderly men, with a mean age in those affected of 72-74 years. Approximately 85% of new prostate cancer diagnoses are made in men over 65 years of age [Grönberg, 2003]. Historical *post mortem* studies have shown that the incidence of indolent, or sub-clinical disease is much greater than this, with 50% of 50-year-old men and up to 80% of 80-year-old men having histological evidence of prostate cancer [Sakr et al., 1996]. The incidence of prostate cancer in the UK has steadily risen over the past few decades; partly as a consequence of *ad hoc* serum prostate specific antigen (PSA) testing and improved diagnostics, but also as a result of a genuine increase in disease incidence [Center et al., 2012; Mayor, 2012; Potosky et al., 1995]. Despite its increasing incidence, mortality from prostate cancer in the UK has been in decline for the past 10 to 15 years [Center et al., 2012].

1.1.2 Risk factors

There are a number of well-established risk factors for the development of prostate cancer [Heidenreich et al., 2011]. It is recognised that men with a first-degree relative with prostate cancer have a two-fold increase in risk over the general population. If more than 2 first-degree relatives are affected, the risk increases by up to eleven-fold [Grönberg et al., 1996; Steinberg et al., 1990]. Approximately 9% of men have true hereditary prostate cancer, defined as three or more affected family members, or at least two who have developed disease before the age of 53 years [Carter et al., 1992]. Prostate cancer incidence has been shown to have greater concordance between monozygotic than heterozygotic twins, indicating a strong influence of genetic factors [Ahlbom et al., 1997; Grönberg et al., 1994].

Some studies have shown familial association between the incidence of prostate cancer and female breast cancer [Bratt, 2000]. Some of this may be explained by the increase in risk of both these cancers associated with germ-line mutations of *BRCA1* and *BRCA2* genes. The risk of prostate cancer for carriers of mutations in either of these genes is increased three-fold as compared with the general population [Easton et al., 1997; Ford et al., 1994; Thorlacius et al., 1998]. Furthermore, *BRCA1* and *BRCA2* mutations are associated with development of more aggressive prostate cancer, with increased likelihood of lymph node spread and distal metastases [Castro et al., 2013]. Mutations of DNA mismatch repair genes, associated with Lynch syndrome have also been shown to increase the risk of developing prostate cancer by three-fold over the general population [Ryan et al., 2014] and are a risk factor for the development of aggressive, metastatic disease [Pritchard et al., 2016].

The risk of prostate cancer is up to 80% greater in men with a history of inflammation or infection of the prostate (prostatitis) [Dennis et al., 2002; Jiang et al., 2013]. This may simply reflect the consequences of increased medical investigation in this patient cohort, however, inflammation is thought to have a direct impact on prostate cancer development and progression [Celhay et al., 2010; Grubisha and DeFranco, 2013]. The potential mechanisms of this effect are discussed

further in section 1.5.1. The other principle risk factors for prostate cancer are age, ethnicity, diet and obesity. These are discussed in section 1.3.1.

1.1.3 Disease presentation

As a result of the prostate's location deep within the pelvis combined with the fact that prostate cancer tends to arise on the outer, posterior aspect of the prostate (the peripheral zone) [Heidenreich et al., 2011; McNeal, 1988], many men with localised prostate cancer do not have any signs or symptoms attributable to the disease. Occasionally, localised disease in the centre of the prostate (transition zone) may give rise to lower urinary tract symptoms that cause difficulty with passing urine (Figure 1.1). New onset of erectile dysfunction has also been shown to be an important symptom of early prostate cancer [Hamilton et al., 2006]. Instead, most localised prostate cancer is detected as a consequence of men undergoing PSA testing, or in men who have prostate tissue removed surgically to relieve urinary symptoms where cancer is then detected incidentally [Center et al., 2012]. In the later stages of the disease, men may present with bone pain arising from metastatic disease or unexplained weight loss [El-Amm and Aragon-Ching, 2016; Hamilton et al., 2006].

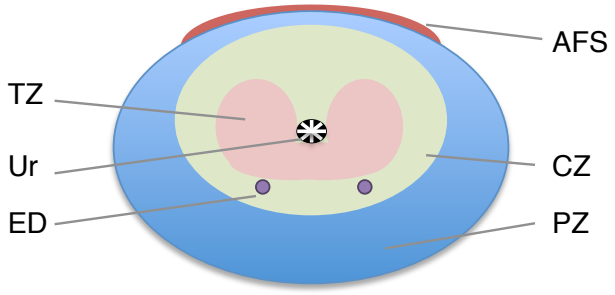


Figure 1.1: **The zonal anatomy of the prostate gland.** Schematic diagram of an axial section through the mid-prostate, based on McNeal's description of histological zones [McNeal, 1988]. Prostate cancer usually arises in the peripheral zone (PZ) and does not impinge upon the urethra (Ur) to cause urinary symptoms. Benign enlargement of the prostate however, typically arises from the transition zone (TZ) to impair urinary function. AFS, anterior fibromuscular stroma; CZ, central zone; ED, ejaculatory duct.

1.1.4 Risk stratification in prostate cancer

The disparity between the incidence of prostate cancer, and mortality from prostate cancer highlights that a large proportion of prostate cancer is clinically indolent (i.e. men die *with* the disease, rather than *from* it). Conversely, in its most aggressive form, untreated prostate cancer has a mortality rate approaching 80% [Bill-Axelson et al., 2014]. Currently in the UK, risk stratification in prostate cancer is based on the D'Amico system, which includes serum PSA, histological grade of cancer (Gleason grade) and clinical stage of the disease (using the Tumour, Nodes, Metastases (TNM) system) as determined by digital rectal examination and cross-sectional imaging such as Computed Tomography (CT) or Magnetic Resonance Imaging (MRI) scan (Table 1.1) [D'Amico et al., 1998; Graham et al., 2014; Heidenreich et al., 2014].

The Gleason score is an assessment of the degree of de-differentiation of prostate tissue as determined by the histological architecture of the tissue and a number of cytological features [Gleason, 1966]. The original description graded cancer from 1 - 5, however as Gleason 1 and 2 are no longer regarded as malignant, this was revised to a grading from 3 - 5 [Epstein et al., 2005]. Grade 3 prostate cancer is well differentiated, whereas grade 5 is poorly differentiated. The Gleason sum score is generated from adding the two most predominant areas of histology, or the predominant area plus the highest grade minority area to give a sum score of between 6 and 10.

Using the D'Amico stratification, men are grouped into low, intermediate or high risk disease at the time of diagnosis. The longest currently available follow up data of untreated prostate cancer has demonstrated that cancer specific survival (CSS) for low risk disease is 86%, whereas for high risk disease it is 64% at 18 years follow up [Bill-Axelson et al., 2014].

1.1.5 Treatment of localised prostate cancer

There are multiple established treatments for prostate cancer. The principle determinants of which treatments are suitable are the D'Amico risk profile and

A

Clinical T staging – Primary tumour		
Tx		Primary tumour cannot be assessed
T0		No evidence of primary tumour
T1		Clinically inapparent tumour not palpable or visible by imaging
	T1a	Incidental finding in ≤5% resected tissue
	T1b	Incidental finding in >5% resected tissue
	T1c	Tumour identified by needle biopsy
T2		Tumour confined to the prostate
	T2a	Tumour involves up to one half of one lobe
	T2b	Tumour involves more than half of one lobe
	T2c	Tumour involves both lobes
T3		Tumour extends through prostatic capsule
	T3a	Extracapsular extension
	T3b	Tumour invades seminal vesicles
T4		Tumour is fixed or invades adjacent structures other than seminal vesicles

B

Level of Risk	PSA (ng\ml)		Gleason sum score		Clinical Stage
Low	<10	and	<7	and	cT1c
Intermediate	10-20	or	7	or	cT2b-2c
High	>20	or	8-10	or	≥cT3a

Table 1.1: **Clinical risk stratification in localised prostate cancer** is based on the clinical tumour stage as determined by the TNM classification (A) in conjunction with the serum PSA level and Gleason score. These are combined into the D'Amico classification (B), which stratifies men into low, intermediate or high risk disease. These are the parameters currently recommended by the European Urological Association (EAU) [Heidenreich et al., 2014].

the general medical health (often known as performance status) of the individual patient (Table 1.2). In UK practice, the patient's own preferences are also an important factor. A detailed discussion of the clinical management of prostate cancer is beyond the scope of this thesis, however a brief summary is presented below with the aim of establishing the clinical context for the work presented in this study.

	Low	Intermediate	High
Watchful Waiting	option	option	option
Active Surveillance	preferred	option	not recommended
Brachytherapy	option	option	with EBRT
Radical Prostatectomy	option	preferred	preferred
Radical Radiotherapy	option	preferred	preferred

Table 1.2: **Summary of treatment options for localised prostate cancer,** as determined by D'Amico risk stratification and patient performance status. EBRT; external beam radiotherapy. Summarised from NICE Guidance (www.nice.org.uk/guidance/CG175).

Watchful waiting

It is well-established that in order for a man to expect to benefit from radical (i.e. curative) treatment of prostate cancer, he must have a life expectancy of at least 10 years from the time of diagnosis [Bill-Axelson et al., 2014]. For those men who do not have a life expectancy of 10 years (as a consequence of advanced age or medical comorbidities) and are asymptomatic at the time of disease presentation, a 'watchful waiting' approach may be used. Men receive no treatment unless they develop symptoms related to prostate cancer progression such as lower urinary tract symptoms or pain. If symptoms develop, then they are treated palliatively, with the aim of symptom control, rather than cancer cure [Heidenreich et al., 2014].

Active surveillance

Active surveillance aims to address the problems of over-diagnosis and over-treatment of prostate cancer, by accounting for the fact that a proportion of histologically diagnosed prostate cancer is clinically indolent, and will not progress to manifest in clinical symptoms in the course of the patient's lifetime [Heidenreich et al., 2014]. Men with low, or selected men with intermediate risk disease, who are suitable for radical therapy, do not receive immediate treatment, but instead enter a program of careful monitoring with repeated serum PSA measurements and prostate biopsies. In this way, if their disease does not progress then the patients do not incur the risks associated with unnecessary treatment, but if progression does occur, then it can be detected and treated radically in a timely manner. Clinical outcomes of active surveillance programs are very favourable, with 5-year cancer specific survival rates in low risk disease of 97-100% [Dall'Era et al., 2008; Klotz et al., 2010; Soloway et al., 2010; Tosoian et al., 2011; van As et al., 2008; van den Bergh et al., 2009].

Brachytherapy

Brachytherapy is a form of localised radiotherapy, which in prostate cancer is delivered by two methods. Low dose rate brachytherapy involves permanent implantation of radioactive seeds ($Iodine^{125}$) into the prostate, whereas in high dose rate brachytherapy $Iridium^{192}$ wires are placed into the prostate transperineally for several hours to deliver a predetermined dose of radiotherapy. Brachytherapy is used in low and intermediate risk disease, and combined with external beam radiotherapy (EBRT) in high risk disease. It is contraindicated in men with large prostates or lower urinary tract symptoms due to the risk of urinary retention following tissue implantation. Five-year progression-free survival is 80-85% and 50% for low and intermediate risk disease respectively [Ragde et al., 2000].

Radical prostatectomy

Radical prostatectomy (RP) is the surgical removal of the entire prostate gland and seminal vesicles, from the external urethral sphincter at the level of the perineal membrane, to the bladder neck. The founding principles for the operation in

its current form were established following landmark work by Walsh and Donker [Walsh, 1980; Walsh and Donker, 1982]. The procedure may be performed by open surgical, laparoscopic and robot-assisted laparoscopic approaches [Heidenreich et al., 2014]. Randomised trial data has shown that RP reduces the risk of death from prostate cancer (compared with no treatment) by 50%, and the greatest benefit is seen in younger men with intermediate risk disease [Bill-Axelson et al., 2014]. Recently published data has shown that increasing numbers of men with high risk disease are undergoing RP with oncological outcomes equivalent to those achieved previously for low and intermediate risk disease, demonstrating ongoing improvement in surgical technique and increasing surgeon confidence in operating on high risk men [Gnanapragasam et al., 2016].

Radical radiotherapy

Radical radiotherapy (RRT), also known as External Beam Radiotherapy (EBRT) to differentiate it from brachytherapy can be used in men suitable for radical treatment, with low, intermediate or high risk disease. The current gold standard is Intensity Modulated Radiotherapy (IMRT), which uses 3D anatomical imaging to map the radiation beam to the prostate in real time [Heidenreich et al., 2014]. A typical course of IMRT for localised prostate cancer delivers a radiation dose of at least 74 Gy. Combining RRT with androgen deprivation therapy (ADT) (section 1.1.6) significantly improves oncological outcomes [Widmark et al., 2009].

1.1.6 Treatment of advanced prostate cancer

Androgen deprivation therapy

Men presenting with prostate cancer unsuitable for radical treatment either due to poor performance status or because the disease has spread from the prostate into surrounding structures (locally advanced) or to distant sites (metastasis) will typically receive ADT as first-line treatment. ADT was developed as a direct result of the landmark discovery by Huggins and Hodges that prostate cancer is an androgen-regulated and androgen-driven disease [Huggins, 1942, 1943; Huggins and Clark, 1940] (section 1.2.1). The mainstay of ADT is leutinising

hormone-releasing hormone (LHRH) analogues, which suppress testicular androgen production by positive-feedback inhibition of the hypothalamo-pituitary-gonadal (HPG) axis [Huggins and Hodges, 1972]. LHRH analogues may be used in combination with androgen receptor (AR) antagonists such as Bicalutamide to achieve complete androgen blockade [Heidenreich et al., 2014]. ADT is also used to treat men with biochemical relapse (BCR - defined as two consecutive increases in serum PSA $\geq$0.2 ng/ml [Heidenreich et al., 2014]) or metastatic recurrence following radical therapy.

Treatment of castrate-resistant prostate cancer

Unfortunately, despite the fact that most men initially respond to ADT, inevitably the tumours become resistant to this treatment leading to BCR and ultimately clinical progression, at which point the disease is termed castrate-resistant prostate cancer CRPC [Scher et al., 2004] (section 1.2.1). CRPC carries a poor prognosis, with median survival of 18 months from diagnosis [Wu et al., 2007]. First-line treatment of CRPC is with docetaxel chemotherapy, which inhibits mitosis by blocking microtubule assembly. Fifty-three percent of patients see a 50% reduction in serum PSA, and overall survival is improved by approximately 2.5 months [Shelley et al., 2006]. For men who develop docetaxel resistance, newer AR-targeting agents such as abiraterone and enzalutamide are available and have been shown to improve prostate cancer survival [de Bono et al., 2011; Scher et al., 2012], however, they are not curative [Lamb et al., 2013].

1.1.7 A clinical rationale for biological research in prostate cancer

There is an ongoing and pressing need to further our understanding of the underlying biology that drives the initiation and progression of prostate cancer, and how its natural history might be modified in the early stages of this process in order to improve clinical outcomes. This is highlighted by two key clinical sequelae of prostate cancer: i) BCR occurs in 20-50% of patients despite radical therapy [Paller and Antonarakis, 2013] and ii) all AR-targeted therapies (ADT, Enzalutamide, Abiraterone) drive a selection pressure, which results in prostate cancer

eventually developing resistance to these treatments [Claessens et al., 2014; Giacinti et al., 2014; Scher et al., 2004]. It has become apparent that alternative strategies to treating prostate cancer are required that do not focus directly on the AR. Recent work on metabolic pathways [Jurmeister et al., 2014; Kaushik et al., 2016; Pertega-Gomes et al., 2015] and immune pathways [Carosella et al., 2015] that influence AR function are such examples. The aim of this approach is to find ways of modulating the influence of AR in prostate cancer initiation and progression so that the selection pressure of direct AR-targeting therapies is slowed or avoided [Mills, 2014].

To that end, understanding the biology and targeting the effects of estrogen in prostate cancer are attractive propositions as estrogen has been shown to have an essential role in prostate cancer development and progression, and drugs aimed at estrogen-dependent targets are already in clinical use for the treatment of breast cancer. Historically, the first approach to hormone treatment of prostate cancer used systemic estrogen therapy in the form of diethylstilbestrol (DES) [Huggins and Hodges, 1972]. This suppressed androgen production indirectly via negative feedback inhibition of the HPG axis. Although still used as a second- or third-line hormonal treatment, DES has fallen out of widespread clinical usage because of the unacceptably high rate of cardiovascular side effects [Morales and Pujari, 1975]. More recently however, it has been found that parenteral administration of estrogens using a transdermal patch avoids first-pass liver metabolism and is not associated with the cardiovascular toxicity of enterally administered estrogens [Langley et al., 2013]. Whilst this important finding addresses the issue of estrogen-induced cardiovascular toxicity, any estrogen-derived prostate cancer treatment must account for the potentially differing roles of the two estrogen receptors in order to be beneficial.

1.2 Steroid hormone receptors in prostate cancer

The sex steroid hormone receptors AR, estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) are Type I nuclear hormone receptors and members of the nuclear hormone receptor superfamily. They are characterised by common structural elements, composed of four functional domains. The N-terminal domain (NTD) followed by the DNA-binding domain (DBD), the C-terminal ligand-binding domain (LBD) and the hinge domain, which connects the DBD to the LBD and is important for the process of nuclear localisation (Figure 1.2). In the absence of ligand, these receptors (with the exception of ERα) are typically located in the cell cytoplasm bound to a chaperone complex. Upon binding of ligand to the LBD, these receptors are translocated to the cell nucleus to bind to DNA and activate transcription of target genes [Mangelsdorf et al., 1995].

1.2.1 The androgen receptor

One of the earliest observations suggesting a role for androgens in regulation of the prostate gland came from John Hunter in 1786 [Hunter, 1786]. He found that the prostates of male animals such as roe deer were substantially greater in size and more mucinous during the rutting season than those of animals observed in winter. Subsequently, the landmark research of Huggins and Hodges demonstrated conclusively that the prostate increased or decreased in size following administration of testosterone or surgical castration respectively [Huggins, 1942, 1943; Huggins and Clark, 1940]. They went on to show that administration of systemic estrogens suppressed androgen production via the HPG axis to suppress prostate cancer growth [Huggins and Hodges, 1972], forming the basis for ADT.

The *AR* gene is located on the X-chromosome between Xq11 and Xq12 [Brown et al., 1989; Migeon et al., 1981], and contains a protein coding region of eight exons. The AR protein consists of 919 amino acids and has a molecular weight of 110 kDa [Gelmann, 2002] (Figure 1.3). Unliganded AR is located in the cell cytoplasm, bound to the chaperone complex made up many proteins includ-

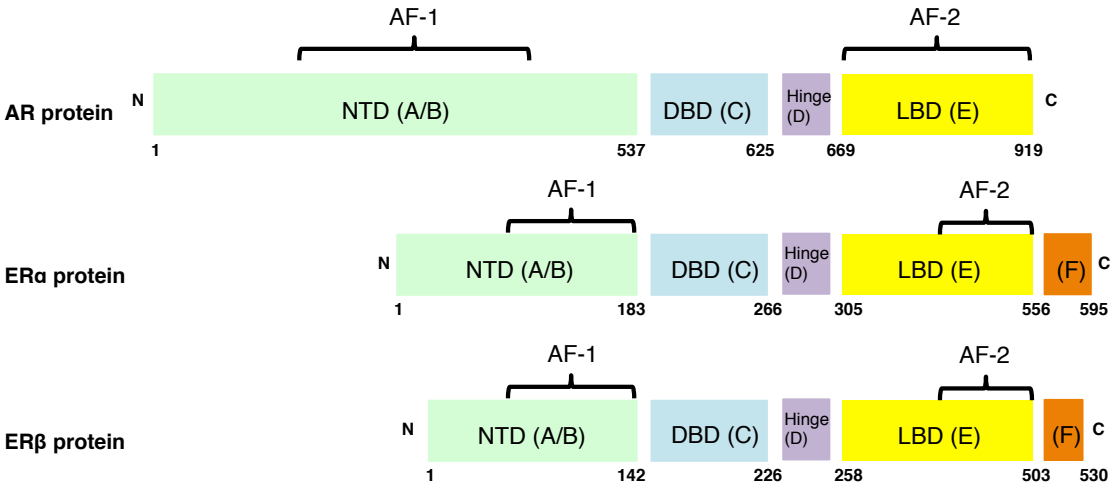


Figure 1.2: **Structure of nuclear steroid hormone receptors.** As with other type-I nuclear receptors, AR, ERα and ERβ are composed of domains A-E, which contain the N-terminal domain (NTD), DNA-binding domain (DBD), hinge domain and ligand binding domain (LBD). The LBD consists of 12 alpha-helices, which allow for ligand docking. The role of the F-domain in ERα/β is less well-defined. The location of activation function (AF) 1 and 2 domains is shown. Numbers below each schematic indicate the amino acid position of each domain (Figure adapted from Hewitt and Korach, 2002; Lorente et al., 2015; Tan et al., 2015).

ing HSP90, FKBP5 and FKBP4. Upon ligand activation with the potent androgen 5α-dihydrotestosterone (DHT), AR decouples from the chaperone complex, translocates into the cell nucleus and undergoes dimerization. The DBD then recognises and binds to androgen response elements (ARE) on DNA, which are typically located in promoter and enhancer regions of AR-regulated genes [Claessens et al., 1996; Schoenmakers et al., 2000; Verrijdt et al., 2000].

AR is a key factor that plays an essential role in all stages of prostate cancer development and progression [Pomerantz et al., 2015; Scher et al., 2004], although much less is known about the role of AR in the initiation of prostate carcinogenesis [Zhou et al., 2015]. AR is required for the normal *in utero* development and subsequent homeostasis of the prostate, where it maintains differentiation of luminal epithelial cells. In order for prostate cancer to develop, it has been proposed that

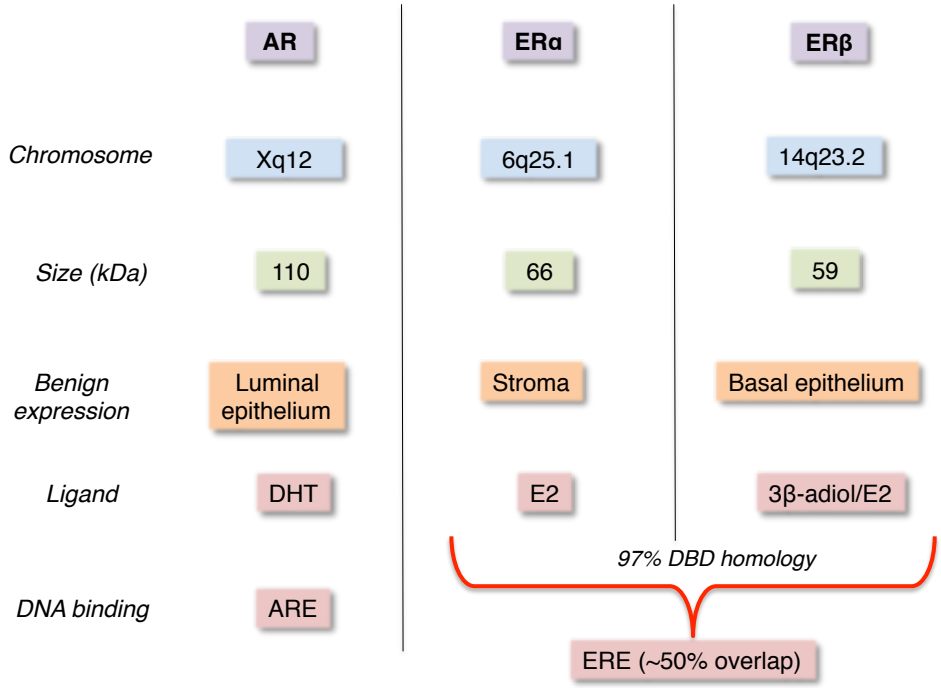


Figure 1.3: **Key characteristics of steroid hormone receptors in prostate cancer,** showing the gene loci, protein size and typical expression in benign prostate tissue. Binding of the receptor with its principle ligand results in translocation to the cell nucleus, DNA binding at ARE or ERE and transcription of target genes. E2, estradiol; ERE, estrogen response element; ARE, androgen response element; ERα, estrogen receptor alpha; ERβ, estrogen receptor beta.

the role of AR must therefore 'switch' from a homeostatic to an oncogenic one [Zhou et al., 2015]. The mechanism of this switch is as yet undefined, though it has been proposed that multiple processes such as post-translational modifications to AR [Coffey and Robson, 2012; Gioeli and Paschal, 2012], aberrant expression of AR co-factors in response to DNA damage and oxidative stress [Mills, 2014] or chronic exposure to low serum testosterone levels as occurs with advancing age, all result in upregulation of AR expression [Zhou et al., 2013]. Recent work has shown that two co-transcription factors FOXA1 and HOXB13 act to reprogram AR genome-wide binding (the cistrome) from a benign to a cancer-associated AR cistrome. This altered binding profile then drives the transformation of epithelial cells from a benign to malignant phenotype [Pomerantz et al., 2015].

The majority of prostate tumours will initially respond to ADT as evidenced by tumour shrinkage and decrease in serum PSA. Inevitably, however after a period of time prostate cancer develops resistance to ADT to become CRPC [Scher et al., 2004]. Although resistant to hormonal manipulation, CRPC is still characterised and driven by AR signalling. Mechanisms accounting for this include amplification or mutation of the AR gene, stabilisation of AR protein, altered expression of AR coregulators, generation of constitutively active AR splice variants and increased synthesis of intra-tumoural androgens [Bubendorf et al., 1999; Cai and Balk, 2011; Chmelar et al., 2007; Dehm et al., 2008; Holzbeierlein et al., 2004; Hu et al., 2012; Ishizaki et al., 2013]. Thus, whilst CRPC is characterised by resistance to manipulation of androgens, it remains androgen receptor dependent.

1.2.2 The estrogen receptors

Both ERα and ERβ are expressed in prostate tissue. It is generally thought that they have opposing roles with respect to prostate cancer with ERα being predominantly oncogenic, promoting cell proliferation and survival and ERβ being predominantly protective, anti-proliferative and pro-apoptotic (reviewed in [Nelson et al., 2014]). ERα and ERβ share substantial sequence homology, with 97% of the DBDs and 60% of the LBDs being identical between the two receptors [Hewitt and Korach, 2002]. This allows both ERα and ERβ to recognise and bind to estrogen response elements (ERE) on DNA with equal affinity [Kuiper et al., 1996; Le et al., 2013].

Estrogen receptor beta

ERβ was first identified in the rat prostate [Kuiper et al., 1996]. Human ERβ is a 59 kDa protein encoded by eight exons of the *ESR2* gene, located on chromosome 14 (Figure 1.3) [Enmark et al., 1997]. The principle ligand of ERβ is 5α-androstane-3β,17β-diol (3β-adiol), which is formed from metabolism of DHT by prostatic 3β hydroxysteroid dehydrogenase [Oliveira et al., 2007; Steckelbroeck et al., 2004; Weihua et al., 2002a,b]. Intraprostatic production of 3β-adiol is reported to be high in benign prostate tissue [Piccolella et al., 2014] and 3β-adiol

has been shown *in vitro* to inhibit prostate cancer cell migration by ERβ-mediated induction of E-cadherin expression [Guerini et al., 2005].

ERβ has been shown to bind to DNA as both a ERβ/β homodimer, and ERα/β heterodimer [Pettersson et al., 2000]. In this heterodimer configuration, ERβ is thought to act as a dominant negative regulator of ERα, acting to modulate transcriptional responses to estrogens in a tissue-dependent manner [Bottner et al., 2014; Pettersson et al., 2000]. ERβ is expressed in a wide range of reproductive and non-reproductive tissues including the central nervous system, cardiovascular system, gastrointestinal tract, urogenital tract (male and female) and skeleton [Bottner et al., 2014; Enmark et al., 1997]. The physiological role of ERβ in each of these tissues has not been fully determined, but it has been implicated in glucose and insulin homeostasis, and may also modulate immunologically-mediated inflammatory pathways [Foryst-Ludwig et al., 2008; Harris et al., 2003].

Estrogen receptor beta isoforms

At least five isoforms of ERβ have been identified [Leung et al., 2006b]. Wild type (wt) ERβ is composed of eight exons, the first six of which are common to the five isoforms. The functional domains A-D (which includes the DBD) are located in these conserved exons, but the LBD differs between the isoforms (Figure 1.4). Expression of ERβ3 is limited to the testis [Moore et al., 1998] and in ovarian carcinoma, levels of ERβ5 mRNA are increased as compared with benign tissue [Suzuki et al., 2008].

ERβ2, ERβ4 and ERβ5 have all been shown to be expressed in prostate tissue, particularly ERβ2, which has been implicated as an oncogene in high grade and late-stage disease [Chen et al., 2009]. In prostate cancer, increased ERβ2 and ERβ5 expression has been shown to correlate with poor prognosis, BCR and decreased time to metastasis following radical prostatectomy [Dey et al., 2012; Leung et al., 2010]. ERβ2 is the principle isoform seen in prostate cancer; it is thought to act as a dominant negative regulator of wtERβ by forming wtERβ/ERβ2 heterodimers. As ERβ2 lacks a functional LBD, this prevents

ligand activation of wtERβ and disables its tumour-suppressive effect [Cotrim et al., 2013; Leung et al., 2006b]. Furthermore, ERβ2 has been shown to regulate genes involved in epithelial-mesenchymal transition (EMT) such as *TWIST1* and *RUNX2*, which may increase the metastatic potential of tumour cells [Dey et al., 2012; Leung et al., 2010].

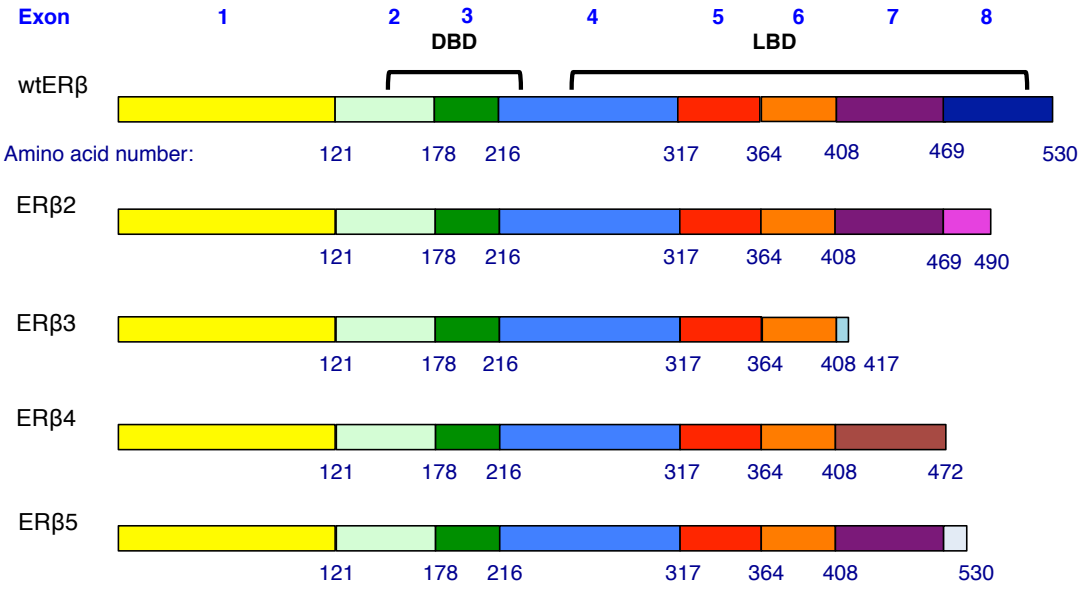


Figure 1.4: **ERβ isoform structure.** Wild type (wt) ERβ is composed of 8 exons. Exons 1 - 6 are shared between the isoforms, but the LBDs differ. (Figure adapted from Nelson et al., 2014).

Estrogen receptor alpha

The gene coding ERα (*ESR1*) is located on chromosome 6 (Figure 1.3). It is a 66 kDa protein, which is structurally similar to other nuclear receptors except for an extra F-domain at the carboxy terminal of the protein [Montano et al., 1995] (Figure 1.2). ERα has been well-characterised and is known to be a principle driver of female breast cancer, in a manner analogous to the AR in prostate cancer [Curtis et al., 2012]. It is less well-known however, that ERα also has a role in the development of prostate cancer.

In utero studies of the developing rodent prostate have shown that ERα expression precedes that of ERβ, and excessive estrogenisation of the prostate mediated by ERα results in squamous metaplasia, inflammation and epithelial dysplasia. This 'imprinting' later manifests as increased risk of prostate cancer development [Arai et al., 1978; McPherson et al., 2008; Prins and Birch, 1997; Prins and Korach, 2008; Prins et al., 2006, 2007]. In benign prostate tissue, ERα is expressed in the prostatic stroma, but not in the epithelial compartment [Tilley et al., 1985; Wernert et al., 1988]. In cancer however, there is evidence of epithelial ERα expression in high-grade prostatic intraepithelial neoplasia (PIN), intermediate- and high-grade tumours and in CRPC [Bonkhoff and Berges, 2009; Celhay et al., 2010; Nelles et al., 2011]. Although not fully understood it has been shown that increased risk of CRPC progression correlates with increased expression of ERα and aromatase, and polymorphisms of aromatase [Celhay et al., 2010; Sissung et al., 2011]. The role of ERα with respect to inflammatory pathways in prostate tissue will be discussed in section 1.5.1.

1.3 The role of estrogen in prostate cancer

1.3.1 Epidemiological evidence

Age and racial factors

As highlighted by the epidemiology of prostate cancer, the primary risk factor for the disease is increasing age [Heidenreich et al., 2011]. It has been observed that the peak age of onset of prostate cancer coincides with the age at which serum testosterone levels begin to decline, whilst serum estrogen levels remain relatively constant [Vermeulen et al., 2002]. This has led to the hypothesis that it is the ratio of estrogen to testosterone (and other circulating androgens) that determines the risk of prostate cancer, rather than the absolute levels of either hormone in isolation [Bosland, 2013; Ellem and Risbridger, 2007]. Indeed, numerous studies have failed to demonstrate a correlation between absolute levels of serum androgens or estrogens and prostate cancer risk [Bosland, 2013; Ross et al., 1992; Yao et al., 2011].

This understanding of circulating hormone levels and prostate cancer risk is of further importance when examining the relationship between race and prostate cancer risk. It is well established that Caucasian men have a lower risk of developing prostate cancer than men of African descent, and that for Japanese men, the lifetime risk of developing prostate cancer is lower still [de Jong et al., 1991; Ellem and Risbridger, 2007; Ross et al., 1992]. Whilst there have been no significant differences in levels of circulating androgens between these three ethnic groups [Ross et al., 1992], levels of serum estrogens have been reported to be greater in black men of African descent than Caucasians [Abd Elmageed et al., 2013; Rohrmann et al., 2007], giving additional credence to the estrogen:androgen ratio hypothesis. Additionally, racial differences in ERβ tissue expression as determined by IHC have demonstrated greater frequency of ERβ staining in black men of African descent than Caucasian men, which in turn correlated with poorer clinical outcome [Abd Elmageed et al., 2013]. It has been proposed that the increased risk of prostate cancer in black men may be partly explained by greater *in utero* estrogen exposure secondary to higher levels of maternal estrogens in black women [Henderson et al., 1988; Nelles et al., 2011; Powell and Meyskens, 2001], resulting in the previously discussed embryological 'imprinting' [Arai et al., 1978; McPherson et al., 2008; Prins and Birch, 1997; Prins and Korach, 2008; Prins et al., 2006, 2007].

Dietary factors and obesity

Estrogen-related dietary factors are also thought to be important in modulating prostate cancer risk [Hori et al., 2011]. Japanese men have a very low incidence of prostate cancer [de Jong et al., 1991], and it is thought that the traditional Japanese diet, which is very rich in phytoestrogens may be protective against prostate cancer. Phytoestrogens and other dietary estrogens such as lignans, flavonoids and lipoflavonoid are thought to have up to 30-fold greater affinity for ERβ than ERα [Ellem and Risbridger, 2007; Kuiper et al., 1998; Thelen et al., 2014]. In prostate cancer cell lines, phytoestrogens have been shown to up regulate ERβ activity, which in turn results in decreased expression of AR [Stettner et al., 2007; Thelen et al., 2005, 2007] and induction of G1-cell cycle block

[Shen et al., 2000]. RNA-sequencing studies conducted in an ERβ-overexpressing MCF-7 breast cancer cell line have shown that the phytoestrogens genistein and S-equol direct ERβ to upregulate genes associated with apoptosis, antiproliferation and DNA-damage response. This is in contrast to estradiol treatment, which promoted proliferation, inflammatory pathways and increased cell motility, thus demonstrating distinctive, ERβ-mediated patterns of gene regulation in response to phytoestrogens [Gong et al., 2014].

Studies in rat models of prostate cancer have demonstrated induction of prostate epithelial cell apoptosis by phytoestrogens [Attia and Ederveen, 2012] and a large epidemiological study has demonstrated reduced prostate cancer incidence in those with a diet rich in phytoestrogens [Hedelin et al., 2006]. It is noteworthy that first- and second-generation immigrants from low-risk countries (such as Japan) to high-risk countries (USA) have a greater risk of developing prostate cancer than age-matched controls in the native country, suggesting that environmental factors such as diet can have a significant influence over an individual's level of risk [Cook et al., 1999].

Obesity is a risk factor for prostate cancer, and is particularly associated with the development of aggressive prostate cancer [Allott et al., 2013; De Nunzio et al., 2013; Vidal et al., 2014]. White adipose tissue, which is predominant in obesity, is a source of estrogen synthesis [Cui et al., 2013]. It expresses significant quantities of aromatase, which is responsible for the conversion of androgens to estrogens [Polari et al., 2015; Wang et al., 2013]. Furthermore, there is a positive correlation between serum estradiol levels and quantity of visceral adipose tissue as measured by MRI [Gautier et al., 2013]. It may be therefore, that the increased risk of prostate cancer associated with obesity is attributable to increased levels of circulating estrogens (with a greater affinity for ERα than ERβ) and disturbance of the previously mentioned estrogen:androgen ratio [Rahman et al., 2016].

1.3.2 Evidence from animal studies

Compelling evidence of the importance of estrogen in prostate carcinogenesis was demonstrated in a series of animal studies, which showed that androgen, estrogen, aromatase and functional ERα are all required for prostate carcinogenesis to occur [Ricke et al., 2008]. Aromatase is expressed in stromal cells of benign prostate [Risbridger et al., 2007], however its expression is upregulated in malignant epithelial cells resulting in increased intraprostatic production of estrogens [Celhay et al., 2010; Ellem et al., 2004]. Expression of aromatase with ERα is an independent predictor of decreased time to BCR in men treated with ADT [Celhay et al., 2010].

To demonstrate the necessity of estrogen in prostate carcinogenesis, Ricke *et al.* administered androgen alone, or androgen and estrogen to aromatase knockout (ArKO) mice [Ricke et al., 2008]. Only the mice receiving both hormones developed prostatic intraepithelial neoplasia (PIN - a premalignant histological phenotype known to be a risk factor for the development of invasive prostate cancer [Merrimen et al., 2009; Nelson et al., 2003]). This lead the authors to conclude that intraprostatic, aromatase-mediated production of estrogens was an important factor in early prostate carcinogenesis (Figure 1.5A). The differential effects of ERβ and ERα were then determined by administering androgen and estrogen to either ERβ knockout (bERKO) or ERα (aERKO) mice (Figure 1.5B). No difference was seen between wild type and bERKO mice, whereas the aERKO mice did not develop PIN, suggesting that ERα is responsible for mediating the harmful, oncogenic effects of estrogen within the prostate gland [Ricke et al., 2008].

Similar studies conducted in rats confirmed these findings [Attia and Ederveen, 2012]. In this study it was shown that testosterone alone was insufficient for PIN to develop; it was only with the addition of a selective ERα agonist (ERα-45) that PIN developed. Furthermore, the addition of an ERβ-selective agonist (ERβ-26) prevented the development of this premaligant phenotype. Taken together, these studies demonstrate firstly, that estrogen is required for prostate cancer to develop

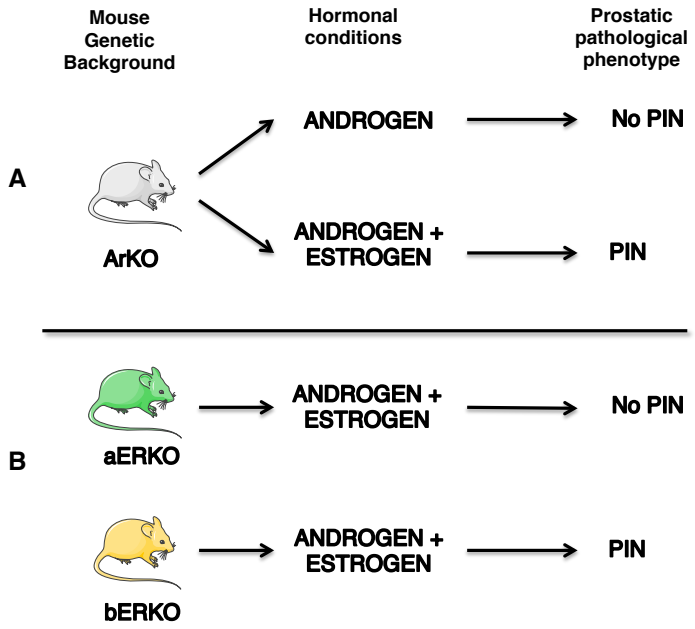


Figure 1.5: **Animal studies showing effects of estrogen and estrogen receptors on prostate carcinogenesis.** Summary of studies conducted by Ricke *et al.* 2008, demonstrating the necessity of aromatase (A), androgen, estrogen and ERα (B) in prostate carcinogenesis. This process is suppressed by functional ERβ. ARKO; aromatase knock-out, aERKO; ERα knock-out, bERKO; ERβ knock-out, PIN; prostatic intraepithelial neoplasia. (Figure adapted from Nelson et al., 2014).

and secondly, that ERα mediates the harmful effects of estrogen in the prostate, whereas ERβ is protective against estrogen-mediated carcinogenesis.

1.3.3 Evidence from preclinical studies of therapeutic compounds

The estrogen receptors represent attractive therapeutic targets as a number of approved drugs are already in widespread use for the treatment of hormone-dependent breast cancer [Lumachi et al., 2011]. The selective estrogen receptor modulator (SERM) raloxifene has been shown *in vitro* to induce apoptosis in androgen-sensitive and androgen-independent prostate cancer cell lines through activation of ERβ, suppression of ERα and induction of caspase-8 and -9 apop-

totic pathways [Kim et al., 2002a,b; Rossi et al., 2011]. A further study showed that raloxifene, tamoxifen (another SERM) and genistein (an ERβ-selective phytoestrogen) reduced growth and proliferation in AR-negative prostate cancer cell lines PC3 and DU145 [Piccolella et al., 2014]. There are however conflicting data in the literature on the *in vitro* effects of genistein, as it has also been reported to promote the development of metastatic disease in an ERβ-dependent manner in mice bearing xenograft tumours [Nakamura et al., 2013]. The ERα antagonist fulvestrant has also been shown to inhibit the growth of prostate cancer cell lines *in vitro* [Lau et al., 2000; Leung et al., 2006a].

Studies conducted in ArKO mice have shown that administration of ERβ-selective agonists induces cystic atrophy in basal cells of prostatic epithelium [McPherson et al., 2010]. These cells express high levels of ERβ, but not AR and are therefore unaffected by conventional ADT [Ruizeveld de Winter et al., 1991]. Thus, once ADT is withdrawn, the prostatic epithelium can be regenerated from this basal cell population. Administration of the ERβ agonist perturbs this process resulting in cellular apoptosis mediated by tumour necrosis factor α (TNFα) in an androgen-independent manner [Hussain et al., 2012; McPherson et al., 2010].

1.3.4 Evidence from drug trials

Despite encouraging preclinical *in vitro* data, as yet there is no clinical trial evidence to support the use of SERMs in prostate cancer. In a promising phase IIb clinical trial of 154 men with biopsy-proven PIN, toremifene (ERα-selective antagonist) was shown to reduce the incidence of invasive carcinoma at 12 months by 48% versus placebo, thus preventing 6.8% of cancers per 100 men per year [Price et al., 2006]. Unfortunately however, a much larger study using toremifene in 1590 men with PIN, with 3 year follow up did not show any statistically significant benefit over placebo [Taneja et al., 2013]. Similarly, fulvestrant [Chadha et al., 2008] and tamoxifen [Bergan et al., 1999] have been shown to be ineffective in the treatment of CRPC.

The reasons underlying the discrepancy between the *in vitro* data and the out-

comes of clinical trials are not presently clear. However, it seems likely that limitations of prostate cancer cell lines used in preclinical studies may be at least partly to blame. None of the prostate cancer cell lines endogenously and simultaneously express ERα, ERβ and AR. Therefore they are not representative of prostate tissue (section 1.4) and can not recapitulate the complex interactions between these three receptors, and other stromal-epithelial interactions relevant to cancer known to occur *in vivo* [Grubisha and DeFranco, 2013; Hanahan and Weinberg, 2011; Madak-Erdogan et al., 2013; Rizza et al., 2014; Robinson et al., 2011; Thelen et al., 2005, 2007; Yang et al., 2012].

1.4 Expression of estrogen receptors in prostate tissue

The reported expression profiles of ERβ and ERα in benign and malignant prostate tissue have strongly contributed to the current paradigm that ERβ is tumour-suppressive and ERα is tumour-promoting in prostate cancer. In benign prostatic tissue, ERα expression is confined to the stromal tissue [Tilley et al., 1985; Wernert et al., 1988]. With the development of cancer however, ERα expression is down-regulated in the stromal cells, and upregulated in malignant epithelial cells [Bonkhoff and Berges, 2009; Celhay et al., 2010; Nelles et al., 2011]. It has been suggested that stromal ERα expressed within cancer associated fibroblasts may even suppress prostate cancer invasion through modulation of macrophage activity [Yeh et al., 2016]. Conversely, stromal ERα expression and elevated aromatase levels have been shown to be independent predictors of shorter time to relapse in CRPC [Celhay et al., 2010], and ERα expression combined with aromatase with the R264C polymorphism have also been linked to decreased progression-free survival in CRPC [Sissung et al., 2011]. A recent study showed that administration of the LHRH antagonist degarelix to men undergoing radical prostatectomy for localised prostate cancer resulted in increased epithelial ERα expression and upregulation of ERα-associated genes, suggesting that ERα expression in prostate tissue may be repressed by AR [Shaw et al., 2016]. Taken together, these data suggest that ERα may have differing roles depending

on specific tissue compartment-dependent contexts, but that when expressed in malignant epithelial cells it may continue to drive prostate cancer progression in castrate conditions.

ERβ is highly expressed in the basal and secretory compartments of benign prostatic epithelium [Horvath et al., 2001]. In malignant prostatic epithelium, however, expression of ERβ is absent [Bonkhoff et al., 1999; Horvath et al., 2001]. It has been reported that ERβ expression negatively correlates with increasing Gleason grade of prostate cancer [Asgari and Morakabati, 2011; Attia and Ederveen, 2012; Dey et al., 2014; Leav et al., 2001] and that its expression is low in high grade PIN [Risbridger et al., 2007], reflecting its premalignant phenotype. Decreased ERβ expression has been shown to result in epithelial de-differentiation and growth of high-grade, aggressive tumours [Mak et al., 2013]. All these data support the notion that ERβ is a tumour-suppressor, the expression of which is lost in the process of malignant transformation of epithelial cells. However, in a study of 159 radical prostatectomy specimens, Horvath *et al.* showed that whilst 75% of tumours in their cohort did not express ERβ, those tumours in which ERβ expression was maintained were associated with decreased relapse-free survival irrespective of tumour grade [Horvath et al., 2001]. In contrast, other studies have demonstrated high ERβ expression in bone and lymph node metastases [Bouchal et al., 2011; Zhu et al., 2004] or that the combination of ERβ expression and AR phosphorylation in hormone naïve (untreated) prostate cancer correlates with poor clinical outcome [Zellweger et al., 2013]. Zellweger *et al.* also reported that increased ERβ expression was associated with higher Gleason grade cancer and greater proliferative activity [Zellweger et al., 2013]. These data present a rather more complex, and perhaps confused picture of what the role of ERβ may be in prostate cancer.

There are a number of potential biological explanations for these apparently conflicting data regarding ERβ tissue expression profiles in prostate cancer. It may be due to varying levels of promoter methylation throughout the carcinogenic process resulting in reversible, stage- and tissue-specific changes in ERβ expression, altering its transcriptional role [Cotrim et al., 2013; Risbridger et al., 2007].

It has also been proposed that maintained ERβ expression in cancer cells may convey a selective advantage to subclones of prostate cancer cells, helping them to metastasise, resulting in continued ERβ expression in metastatic deposits [Zhu et al., 2004]. A further explanation may lie with ERβ isoforms, particularly ERβ2, which is reported to be more highly expressed in high-grade and metastatic prostate cancer [Dey et al., 2012]. As previously discussed (Section 1.2.2) ERβ2 is thought to negatively regulate the tumour-suppressive role of wtERβ through heterodimerisation and prevention of ligand binding [Cotrim et al., 2013; Leung et al., 2006b] and ERβ2 has been implicated specifically in the process of cancer metastasis through upregulation of EMT genes such as *TWIST1* and *RUNX2* [Chen et al., 2009; Dey et al., 2012; Leung et al., 2010]. If the antibodies used for IHC in these studies recognise the protein region common to both wtERβ and specific isoforms then this too may be an explanation for some of the apparently contradictory IHC data described.

It may be however, that the underlying source of these controversies is a technical, rather than a biological one. It is widely known in the field that there is marked variability in the specificity and sensitivity of commercially available ERβ antibodies, which may give rise to inconsistencies in the findings reported between IHC-based studies [Choi et al., 2001; Hartman et al., 2012; Skliris et al., 2002; Weitsman et al., 2006; Wu et al., 2012]. This critically important question is the focus of studies presented in Chapter 3 of this thesis.

1.5 Biology of ERβ in cancer

1.5.1 Estrogens, inflammation and EMT in prostate cancer

Inflammation of the prostate is a recognised risk factor for the development of prostate cancer and cancer in general [Dennis et al., 2002; Hanahan and Weinberg, 2011; Jiang et al., 2013]. Several mechanisms, centring on ER function and linking to EMT-related pathways have been implicated in the development and progression of prostate cancer. EMT is a marker of oncological progression, which

enables cancer cells to invade surrounding tissues and subsequently metastasise to distant sites [Hanahan and Weinberg, 2011]. ERβ is a negative regulator of inflammatory processes [Harris et al., 2003], and in prostate tissue its expression has been shown to correlate with E-cadherin levels [Mak et al., 2010]. Loss of E-cadherin expression is a well-established marker of EMT, and as ERβ expression declines during malignant transformation, E-cadherin levels also decline. In addition, ERβ transcriptional activity is sensitive to inflammation-related oxidation by hydrogen peroxide (H_2O_2) and other reactive oxidation species. This leads to reduced DNA-binding by ERβ resulting in decreased E-cadherin expression [Grubisha et al., 2012]. This results in loss of cell adhesion, increased cell motility and increased propensity for metastastic dissemination of the disease [Grubisha and DeFranco, 2013]. Reactive oxygen species such as H_2O_2 are generated by the pro-inflammatory enzyme cyclo-oxygenase 2 (COX2), which is over-expressed in prostate cancer [Kirschenbaum et al., 2001; Richardsen et al., 2010]. This establishes a pro-inflammatory positive feedback loop (Figure 1.6).

A second pro-inflammatory positive feedback loop, centring on ERα function is also thought to have a role in prostate cancer progression [Ellem and Risbridger, 2007] (Figure 1.6). Expression of aromatase is increased in prostate cancer, which results in raised levels of intraprostatic estrogens that then act via ERα to promote tissue inflammation via local generation of nitric oxide (NO) [Celhay et al., 2010; Ellem and Risbridger, 2007; Nelles et al., 2011; Pinzone et al., 2004; Risbridger et al., 2007]. Pro-inflammatory mediators such as TNFα and prostaglandin E_2 in turn upregulate *CYP19* expression, which increases aromatase activity [Subbaramaiah et al., 2011]. This inflammatory process has been confirmed in mouse models through observation of neutrophil and leucocyte migration from the stroma to the epithelial compartment [Bianco et al., 2002, 2006].

1.5.2 Genomic mechanisms of ERβ

Regulation of ERβ expression

Transcription from the *ESR2* gene is controlled by two gene promoters, 0N and 0K [Lee et al., 2013; Suzuki et al., 2008]. The 0N promoter is regulated epi-

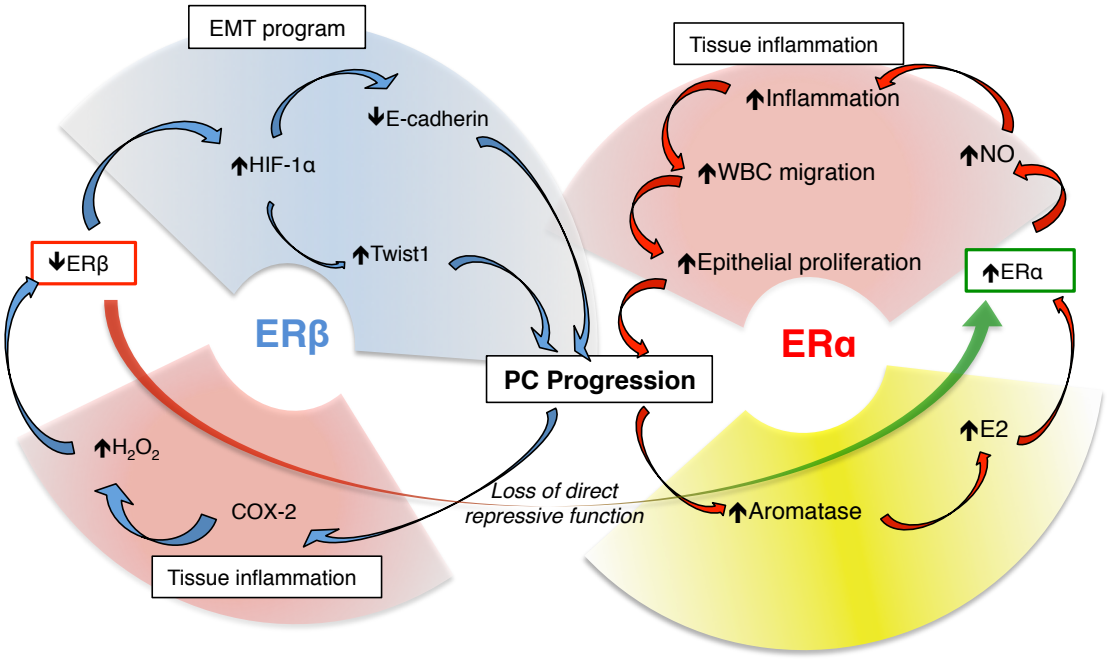


Figure 1.6: **Estrogen-mediated influence of inflammation in prostate cancer.** In response to tissue inflammation, decreased ERβ expression results in up-regulation of the EMT program to promote metastasis. Inflammation also leads to increased aromatase expression and infiltration of inflammatory cells. (WBC, white blood cell; E2, estradiol; NO, nitric oxide; H_2O_2, hydrogen peroxide; COX2, cyclo-oxygenase 2). (Figure adapted from Nelson et al., 2014).

genetically by a CpG island contained within it [Zhu et al., 2004]. Wild type ERβ is transcribed predominantly from the 0N promoter, which has the greater transcriptional activity of the two [Lee et al., 2013; Suzuki et al., 2008]. Several studies have shown that decreased expression of ERβ in cancer is the consequence of DNA-methylation of the 0N promoter [Lee et al., 2013; Rody et al., 2005; Zhao et al., 2003]. In contrast, ERβ isoforms, ERβ2 and ERβ5 are predominantly transcribed from the 0K promoter, which is not hypermethylated, resulting in maintained transcription of ERβ2 and ERβ5 mRNA in cancer [Lee et al., 2013; Suzuki et al., 2008]. It is hypothesised that this is the mechanism by which ERβ2 expression increases in high grade and metastatic prostate cancer [Lee et al., 2013].

The 0N promoter is also thought to be regulated by the transcriptional repressor BMI-1, which is a key component of the polycomb repressive complex [Jacobs et al., 1999]. BMI-1 binds within the 0N, but not the 0K promoter regions of the *ESR2* gene [Mak et al., 2015a]. Expression of BMI-1 is increased following loss of *PTEN*, one of the most common genetic lesions occurring in prostate cancer. Indeed, the frequency of *PTEN* loss increases with Gleason grade and is associated with increasingly aggressive disease [Cairns et al., 1997]. Furthermore, *PTEN* loss results in inhibition of the apoptotic functions of FOXO3a, a forkhead protein, which is upregulated in ERβ-mediated cellular apoptosis via the intrinsic pathway [Dey et al., 2014]. Following BMI-1 induced repression of ERβ expression, HIF1α and VEGF expression are upregulated, which in turn establishes a positive feedback loop to sustain BMI-1 expression [Goel et al., 2012; Mak et al., 2015a]. Taken together, these findings provide insight into some of the early events in prostate carcinogenesis and an important relationship between *PTEN* deletion and silencing of ERβ expression with resultant loss of its tumour-suppressive effect [Mak et al., 2015a] (Figure 1.7).

ERβ-DNA binding

ERβ and ERα both bind to EREs on DNA to activate dependent gene transcription [Kuiper et al., 1996; Le et al., 2013]. The two receptors share 97% sequence homology in the DBD and 60% in the LBD, with the result that there is substantial overlap in their genome-wide DNA binding profiles [Madak-Erdogan et al., 2013]. The binding affinity of each ER to the ERE can be affected by changes in the nucleotide sequence, which results in differential recruitment of co-activators, protein conformation and transcriptional activity. This highlights an important mechanism by which the differential functions of ERβ and ERα are mediated at a genomic level [Klinge et al., 2004].

At present, the genome-wide binding of endogenously expressed ERβ has not been determined from any experimental model, so current understanding is based on studies using exogenous or tagged ERβ in cell line models [Le et al., 2013; Madak-Erdogan et al., 2013; Zhao et al., 2010]. Using the breast cancer cell line MCF-

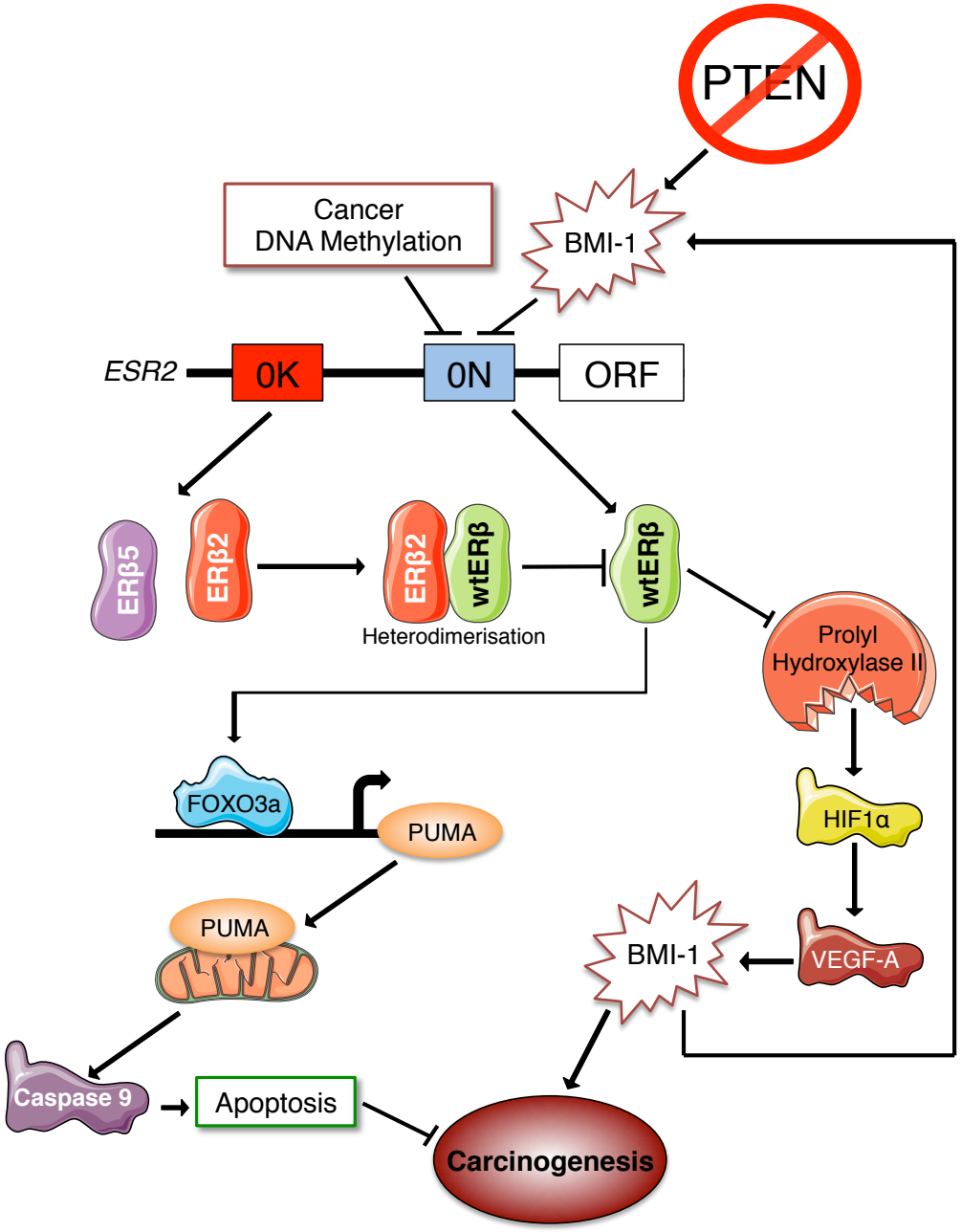


Figure 1.7: **Silencing wtERβ activates multiple carcinogenic mechanisms.** *PTEN* deletion results in disregulation of ERβ's tumour suppressive function by i) Silencing wtERβ expression from 0N promoter whilst maintaining ERβ2 and ERβ5 expression. ERβ2 heterodimerises to inhibit wtERβ function; ii) Loss of wtERβ results in upregulation of VEGF signalling to promote carcinogenesis, establishing a positive feedback loop via BMI-1; iii) Loss of wtERβ disables caspase-9-mediated apoptosis to promote carcinogenesis. (Figure based on Dey et al., 2014; Lee et al., 2013; Leung et al., 2006b; Mak et al., 2015a; Suzuki et al., 2008).

7 with force-expressed ERβ (MCF-7 ERα/β), force-expressed ERβ with ERα knockdown (MCF-7 ERβ) or wild type MCF-7 (MCF-7 ERα), Madak-Erdogan *et al.* showed that the two receptors compete for DNA binding sites, and in doing so, modify each other's transcriptional effects. Specifically, when each of the receptors was expressed alone, there was 40% overlap between ERα and ERβ binding sites. However, when co-expressed (MCF-7 ERα/β cells) the number of binding sites available to each receptor decreased by approximately 50% [Madak-Erdogan et al., 2013]. Additionally, they showed that the proliferative effects of ERα transcription were reduced by the co-expression of ERβ, demonstrating that ERβ can modulate the oncogenic effects of ERα. ERβ has been shown to directly bind to the ERα promoter region to repress ERα expression, providing a mechanistic explanation for this particular observation [Bulun et al., 2012]. By integrating cistromic and transcriptomic analyses, Madak-Erdogan *et al.* showed that ERβ's anti-proliferative effects were mediated through direct binding to apoptosis and cell-cycle regulation genes, and modulation of metabolic pathways through coupling with the coregulator NRIP1 (RIP140) [Madak-Erdogan et al., 2013]. These data, along with similar work by others [Le et al., 2013] support the hypothesis that ERβ is predominantly tumour-suppressive. However, as these studies were conducted in derivatives of breast cancer cell lines, it is important that the hypothesis is further tested in appropriate prostate cancer models.

Genomic crosstalk between ERβ and AR

It is becoming increasingly apparent in the nuclear receptor field that individual receptors do not function in isolation, but rather they influence and modulate each other's actions through a number of mechanisms. This phenomenon is known as 'crosstalk' and is an area of significant research interest and translational relevance. One such example is recent work showing that the glucocorticoid receptor (GR) is expressed in CRPC, and can bypass complete androgen blockade to restore expression of a selection of AR target genes and thus continue to drive prostate cancer progression [Arora et al., 2013]. This has important clinical relevance, as corticosteroids are often used to treat patients with advanced CRPC [Heidenreich et al., 2014]. The findings described by Arora *et al.* suggest that

corticosteroid use may in fact accelerate cancer progression in these patients.

Several studies have demonstrated evidence of interaction between ERβ and AR in breast and prostate cancer models, but there are currently no studies reporting genome-wide ERβ/AR crosstalk at the level of chromatin binding in any cell or tissue context.

Upregulation of ERβ in response to phytoestrogens *in vitro* resulted in decreased expression of AR and PSA (an AR target gene) in a prostate cancer cell line model [Thelen et al., 2007]. Another *in vitro* study showed that ligand-activated ERβ repressed AR-dependent cell proliferation [Weng et al., 2013]. These two studies suggest that ERβ negatively regulates AR activity. AR-driven inflammatory pathways in prostate cancer have been shown to inhibit ERβ's tumour-suppressive effect through oxidation by reactive oxygen species (Figure 1.6) [Grubisha and DeFranco, 2013]. It has been suggested that cross-reactivity of ligands may also influence receptor crosstalk. For example, 3β-adiol, the natural ligand for ERβ and the predominant intra-prostatic estrogen [Oliveira et al., 2007; Piccolella et al., 2014] has been shown *in vitro* to activate mutant AR typically found in CRPC, suggesting that 3β-adiol could continue to drive prostate cancer in the testosterone-deplete conditions of maximal androgen blockade [Mizokami et al., 2004]. It has also been suggested that DHT will directly stimulate ERβ resulting in its recruitment to EREs [Teng et al., 2014], however, given that 3β-adiol is a metabolite of DHT this finding may require cautious interpretation. Interestingly, this same study by Teng *et al.* demonstrated dihydroepiandrosterone- (DHEA - the steroid precursor of androgens and estrogens [Labrie et al., 2005]) and estradiol (E2)-induced recruitment of ERβ to AREs [Teng et al., 2014], suggesting a mechanism by which ERβ directly regulates AR function. As this work was conducted in a liver cell line model, it may be that the findings are not generalisable across differing cell or tissue contexts. Indeed a study conducted in a breast cancer cell line showed that AR, upregulated by a non-metabolisable synthetic androgen ligand binds to an ARE within the ERβ promoter region to increase ERβ expression and inhibit breast cancer cell growth [Rizza et al., 2014].

It is difficult to distill one single, coherent mechanistic model of the relationship between ERβ and AR from the above-mentioned studies, and in particular to work out whether ERβ regulates AR or *vice versa*. This may reflect the differing cell- and tissue-specific contexts in which each study has been conducted. Particularly in the context of prostate cancer, where little is known about the genomic actions of ERβ and its potential effect on AR function at a chromatin level, this is an important area for further investigation.

1.5.3 ERβ as an oncogene

The majority of the evidence discussed thus far holds to the paradigm that in breast and prostate cancer, wtERβ is a tumour suppressor and negative regulator of processes that lead to carcinogenesis (ERβ isoforms excepted; see section 1.2.2). However, there are several studies published in the literature that contradict this notion and implicate wtERβ as an oncogene.

Yang *et al.* proposed a model of wtERβ-mediated, non-androgenic AR signalling, which suggested that E2 stimulated ERβ could continue to drive AR-dependent gene transcription in castrate conditions and thus maintain prostate cancer growth [Yang et al., 2012]. In hormone replete conditions, DHT activates AR, which then binds to AREs to stimulate AR-dependent transcription. However, in the absence of DHT, they propose that E2-stimulated wtERβ binds to the AR co-factor and proto-oncogene PELP1 (proline-, glutamic acid- and leucine-rich protein 1), to form a complex that then binds to AR with PELP1 acting as a bridge between the two nuclear receptors. This complex is then recruited to an ARE, to maintain AR-dependent gene transcription, now under estrogen regulation and thus providing a mechanism for ongoing progression of CRPC.

In a study using IHC of hormone-naïve (HN) and castrate-resistant prostate cancer tissue, Zellweger *et al.* reported high expression of wtERβ in CRPC [Zellweger et al., 2013]. Furthermore, expression of wtERβ in HN tumours was significantly associated with adverse prognosis, particularly when co-expressed with phosphylated AR. In light of their findings, the authors propose that the role of wtERβ

may switch from tumour-suppressor, to tumour-promoting as tumourigenesis progresses. Recently, Lombardi *et al.* found that ligand-activated wtERβ was pro-proliferative in the AR-negative, castrate-resistant PC-3 prostate cancer cell line through upregulation of β-catenin induced Cyclin D2 expression [Lombardi et al., 2016].

1.6 Hypothesis and aims

In summary, the literature discussed thus far highlights several key points; firstly, estrogen is essential for the development of prostate cancer and estrogen exposure is an important epidemiological risk factor for prostate cancer; secondly, most evidence suggests that wtERβ is a tumour-suppressor and acts as a gate-keeper to a number of carcinogenic processes, however there are ongoing controversies regarding the role of wtERβ at various stages of prostate cancer progression; thirdly, there is evidence of relationship and interplay between ERβ and AR, but this has not been thoroughly explored at the genomic level, particularly in the context of prostate cancer.

Based on the hypothesis that **ERβ is an important modulator of prostate carcinogenesis**, the principle aim of this work is to investigate and further understand the influence of ERβ on AR-driven carcinogenesis in prostate cancer. To address this, the following studies have been undertaken and are presented in this thesis:

1. **Validation of ERβ antibodies.**
   A panel of commonly used ERβ antibodies were assessed for ERβ specificity and validated using multiple, independent experimental approaches. This is to establish reliable reagents to be used for subsequent validation and mechanistic experiments.

2. **Establishing an experimental model for the study of ERβ.**
   Prostate and breast cancer cell lines commonly used in previously published studies for the study of ERβ were assessed for ERβ expression using the newly validated antibodies and additional, antibody-independent

approaches. As no ERβ expression was detected in these cell lines, an androgen-sensitive prostate cancer cell line model with stably transfected, inducible ERβ expression was developed and validated for use in *in vitro* mechanistic studies of ERβ function. Differential expression of ERβ in benign versus cancerous prostate tissue was confirmed using validated antibody.

3. **Genomic crosstalk between ERβ and AR in prostate cancer.**
   To investigate the relationship between ERβ and AR, published clinical datasets were interrogated, revealing that increased ERβ expression is associated with improved BCR-free survival in men with prostate cancer. Inhibition of AR signalling *in vitro* by silencing RNA and *in vivo* by ADT increases ERβ expression, indicating that ERβ expression is repressed by AR. Using the androgen-sensitive prostate cancer cell line with inducible ERβ expression, we found that ligand-activated ERβ inhibits proliferation and down-regulates androgen-dependent genes. We identified DNA-binding sites shared by ERβ and AR, suggesting that this antagonism occurs through competition for binding sites. These data reveal the genomic mechanisms by which ERβ modulates AR-driven carcinogenesis for the first time. In future, an ERβ-selective compound may be used to slow prostate cancer progression.

Chapter 2

Materials and methods

2.1 Cell Culture Techniques

2.1.1 Cell lines and culture media

The cancer cell line MDA-MB-231-ERβ with doxycycline-inducible ERβ expression (gift from Dr. J. Hawse, Mayo Clinic, Minnesota, USA) was cultured in Dulbeccos Modified Eagle Medium with F12 supplement (DMEM/F12) with 10% heat-inactivated tetracycline-free foetal bovine serum (FBS), 2mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, 5 μg/ml blasticidin S (to select for the tetracycline repressor) and 500 μg/ml zeocin (to select for the ERβ expression vector).
Prostate cell lines LNCaP (androgen sensitive), C4-2, C4-2b, PC3, DU145, 22Rv1 (androgen resistant) and PNT1a (benign) were obtained from ATCC and grown in RPMI-1640 with 10% heat-inactivated FBS, 2mM L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin. Breast cell line MCF-7 was obtained from ATCC and grown in DMEM supplemented with 10% heat-inactivated FBS, 2mM L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin.
LNCaP-ERβ cell line was cultured in RPMI-1640 with 10% heat-inactivated tetracycline-free FBS, 2mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, 2.5 μg/ml blasticidin S (to maintain selection of the tetracycline repressor) and 250 μg/ml zeocin (to maintain selection of the ERβ expression vector).

2.1.2 Cell Culture

All cell lines were incubated at 37°C with 5% CO_2 and cultured to 80 to 90% confluence. Cells were washed with warmed, sterile phosphate buffer saline (PBS) (137 mM NaCl, 2.7mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4), trypsininsed with 0.05% trypsin-EDTA, neutralised with media and then centrifuged for 3 minutes at 1,300 rpm (Heraeus Megafuge, Thermo Fisher Scientific, Leicestershire, UK). The cell pellets were then resuspended in media and replated at a dilution of 1:2 to 1:10 depending on initial confluency and cell line growth rate.
To prepare frozen stocks of cell lines, $1x10^6$ cells were resuspended in 1ml of 10% dimethyl sulphide (DMSO) and 90% FBS per aliquot. Cells were frozen to -80°C. Frozen cells were revived by rapid thawing to 37°C followed by resuspension in appropriate media after centrifugation. All cells lines were confirmed to be free of mycoplasma infection by RNA capture ELISA. STR genotyping was used to confirm the identity of all cell lines used.
To induce ERβ expression in MDA-MB-231-ERβ and LNCaP-ERβ cells, doxycycline was added at doses of either 0.1 μg/ml or 0.5 μg/ml for 24 hours depending on specific experimental requirements.
Conditioned media for LNCaP-Tet-R and LNCaP-ERβ cells was prepared by adding media to parental LNCaP cells for 48 hours. Media was collected and filtered through a Stericup® 0.22μm pore filter (Millipore UK, Watford, UK), then stored at 4°C.

2.1.3 Creation of the LNCaP-ERβ cell line

The LNCaP-ERβ cell line was generated using the T-Rex™ system (Invitrogen) following a previously described protocol [Monroe et al., 2003]. This approach uses two vectors, the pcDNA6/TR©, which is a regulatory plasmid expressing the tetracycline-repressor (Tet-R) and the pcDNA4/TO©. This contains a CMV promotor driving the expression of ERβ under the control of Tet-operator sequences (pcDNA4/TO©-ERβ vector gifted by Dr. J. Hawse, Mayo Clinic, Minnesota, USA). Treating cells with doxycycline releases the Tet-R from the Tet-operator sequence resulting in expression of ERβ.

Preparation of plasmids

pcDNA6/TR©, pcDNA4/TO©-Luc and pcDNA4/TO©-ERβ plasmids were bulked up using Subcloning efficiency™ DH5α™ competent *E. coli* bacterial cells. For each plasmid, 2 ng of plasmid DNA was added to 50 μl of cells and incubated on ice for 30 minutes. Cells were heat-shocked for 20 seconds at 42°C and returned to ice for 2 minutes. To each tube, 950 μl of prewarmed LB media was added and the tubes incubated at 37°C and rotated at 225 rpm for 1 hour. Twenty microlitres of each transformation was then spread on pre-warmed amplicillin-selective plates, and the plates then incubated overnight at 37°C.

Transfection of Tet-R plasmid into LNCaP cells

The pcDNA6/TR© was linearised via *Fsp1* restriction digest. Plasmid DNA was purified by phenol chloroform cleanup. Fourteen micrograms of plasmid DNA was transfected into genotyped LNCaP cells at a density of 90% confluence using Lipofectamine® 3000 (Thermofisher Scientific, Leicestershire, UK). Cells were incubated overnight at 37°C with 5% CO_2. Twenty-four hours later, the culture media was replaced. Forty-eight hours after transfection, the cells were split to a 1:20 dilution to enable growth of single-cell colonies on a 10 cm^2 dish. Following further overnight incubation, Blasticidin S 5 μg/ml was added to select for cells that had successfully integrated the tetracycline repressor. Fresh culture media and blasticidin was added every 48 hours until discrete colonies of cells were apparent on the petri dish.

Selection of LNCaP-Tet-R clones

With the aid of 4x magnification under a light microscope, individual colonies of cells were trypsinised (5μl trypsin), taken from the 10 cm^2 plate and seeded into 96-well plates. Approximately 80 clones of LNCaP-Tet-R cells were sampled in this manner and cultured in 50% fresh culture media and 50% conditioned media up to 6-well plates.

Luciferase screen of LNCaP-Tet-R clones

Twenty clones were successfully raised to 6-well plates. Cells were counted with the Vi-CELL™ (Beckman Coulter Life Sciences, Indianapolis, USA) and 10,000 cells from each clone seeded into 96-well plates in sextuplicate. Following 48 hours of incubation at 37°C in 5% CO_2 the colonies were transfected with pcDNA4/TO© plasmid containing a cloned *luciferase* gene using Lipofectamine® 3000. Following overnight incubation at 37°C in 5% CO_2 fresh media was added. Doxycycline 0.1 μg/ml was added to 3 wells of each clone for 24 hours to induce *luciferase* expression. Steady Glo® (Promega, Southampton, UK) was added to each well and the resulting fluorescence measured using the PHERAstar® (Thermo Fisher Scientific, Leicestershire, UK).

Zeocin dose response assay in LNCaP-Tet-R cells

As the pcDNA4/TO©-ERβ plasmid contains a Zeocin-resistance gene, a dose response assay was conducted to determine the optimal dose of zeocin, which would kill cells that had failed to integrate the pcDNA4/TO©-ERβ vector. LNCaP-Tet-R cells were seeded into 6-well plates (3x10^5 cells per well) and treated with zeocin at the following doses: 0 μg/ml; 250 μg/ml; 500 μg/ml; 750 μg/ml; 1000 μg/ml; 1250 μg/ml. Fresh media and antibiotic was added every 48 hours and cells examined with microscopy for cell death on day 6. On the basis of this experiment, 1 mg/ml zeocin was subsequently used for selection of the pcDNA4/TO©-ERβ plasmid.

Transfection of ERβ plasmid into LNCaP-Tet-R cells

The pcDNA4/TO©-ERβ plasmid was transfected into the LNCaP-Tet-R clone exhibiting firstly; greatest induction of *luciferase* signal and secondly; the lowest signal from the 'doxycycline-off' condition. Using Lipofectamine® 2000, 2 μg of pcDNA4/TO©-ERβ plasmid was transfected into LNCaP-Tet-R cells at 90% confluence. Following 6 hour incubation at 37°C in 5% CO_2 fresh media was added. The following day, blasticidin 5 μg/ml and zeocin 1 mg/ml were added to the media. Fresh media and antibiotics were added every 48 hours for 6 days.

Cells were then maintained in in tetracycline-free media with 2.5 μg/ml blasticidin and 250 μg/ml zeocin as described in section 2.1.1.

2.1.4 Hormone deprivation followed by ligand treatment

Cells were plated at 50% confluence in growth media as described (section 2.1.1). The following day, cells were washed with PBS and media replaced with phenol red-free RPMI 1640 supplemented with 5% charcoal/dextran-treated, tetracycline-screened FBS (Thermo Fisher Scientific, Paisley UK), 2mM L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin. Cells were maintained in steroid-depleted conditions for 3 days, with fresh media replaced every 48 hours. Hormone treatments consisted of vehicle control (ethanol), synthetic androgen R1881 (1nM) (Sigma-Aldrich Company Ltd. Dorset, UK) and 5α androstane 3β, 17β-diol (3β-adiol) 10 nM (LGC Standards, Teddington, UK).

2.1.5 Cell proliferation assay

Cell proliferation in LNCaP-ERβ+ and LNCaP-ERβ- cells, treated with vehicle, 1 nM R1881, 10 nM 3β-adiol or both R1881 and 3β-adiol was assessed using the IncuCyte^{TM} (Welwyn Garden City, UK). Three biological replicates, each of 4 technical triplicate experiments were set up in 48-well plates with 15,000 cells seeded per well. Cells were hormone-deprived as described (section 2.1.4) for 3 days prior to hormone treatment. Cell confluence in each well was measured daily for 7 days.

2.1.6 Silencing RNA to AR

Genotyped and mycoplasma negative LNCaP cells at 90% confluence were treated with silencing RNA (siRNA) to AR (Dharmacon On Target plus Smartpool, L-003400-00, Lot 131115, ThermoScientific, Leicestershire, UK) or non targeting pool (siNT - D-001810-10-20, Lot 1557666). Transfection was carried out using Lipofectamine® 2000 with 50 nM siRNA. Cells were incubated with siRNA for 6 hours at 37°C in 5% CO_2. Cells were then washed with PBS and fresh growth

media added. Media was refreshed 15 hours later, and the cells harvested in PBS with protease inhibitor after a further 24 hours incubation 37°C in 5% CO_2.

2.2 Quantification of mRNA expression

2.2.1 Preparation of mRNA

Cell lines were harvested for collection of mRNA using the RNEasy® Mini Kit (Qiagen, California USA) according to the manufacturer's instructions. On-column DNase digestion was performed with 20 units of RNase-free DNaseI (Roche Diagnostics GmbH, Mannheim, Germany) for 15 minutes at room temperature to remove contaminating genomic DNA.

2.2.2 cDNA synthesis

RNA was quantified by measuring the absorbance at 260 nm and 280 nm with the NanoDrop® 8000 (Thermo Scientific, Delaware USA). Samples containing 250 ng random primers (Promega, Madison, WI, USA), 1 μg RNA, 1 μl 10mM dNTP mix and water to a total volume of 13 μl were prepared and heated to 65°C for 5 minutes, followed by 1 minute incubation on ice. To each sample 4 μl 5X First-strand buffer, 1 μl 0.1M DTT, 1 μl RNaseOUT and 1 μl SuperScript III reverse transcriptase (RT) (Thermo Fisher Scientific, Leicestershire, UK) were added and incubated at 25°C for 5 minutes then 50°C for 60 minutes followed by inactivation of the reaction by heating at 70°C for 15 minutes. Synthesised cDNA was diluted 1:5 using nuclease-free water and stored at -20°C for subsequent use.

2.2.3 Quantitative RT-PCR (RT-qPCR)

Each RT-qPCR reaction contained 7.5 μl Power SYBR® Green PCR Master Mix (Applied Biosystems, California USA), 0.5 μl of 10 μM primer mix, 2 μl of a 1:5 dilution of cDNA and nuclease-free water to a final volume of 15 μl. Reactions were performed with the Stratagene® Mx3005P RealTime machine in triplicate. Hot-start *Taq* polymerase was heat-activated at 95°C for 10 minutes followed by 40 cycles of 15 seconds at 95°C and 30 seconds at 60°C. Fluorescence was read

in each cycle and a melting curve constructed as the temperature was increased from 65°C to 95°C with continuous fluorescence readings. UBC was used as a control gene to normalise between the samples and relative expression determined using the delta-delta Ct method [Livak and Schmittgen, 2001].

2.2.4 RT-qPCR Primer design

Primers for mRNA RT-qPCR were designed based on published genomic sequences (available from USCS genome browser at http://genome.ucsc.edu/) using the Primer3 software package [Koressaar and Remm, 2007; Untergasser et al., 2012] available at http://bioinfo.ut.ee/primer3-0.4.0/primer3/. Primer sequences are shown in table 2.1.

	Forward	Reverse
ERβ	5' AAAACCGGCGCAAGAGCTG 3'	3' TGCTCGTCGGCACTTCTCTG 5'
AR	5' CTCACCAGCTCCTGGACTC 3'	3' CAGGCAGAAGACATCTGAAAG 3'
UBC	5' ATTTGGGTCGCGGTTCTTG 3'	5'TGCCTTGACATTCTCGATGGT 3'

Table 2.1: **Sequences of primers used in RT-qPCR**

2.2.5 RNA Sequencing

Total RNA was extracted using the method described. Libraries for Illumina sequencing were prepared using TruSeq Stranded mRNA HT kit (Cat. RS-122-2103, Illumina, Chesterford UK). Prior to library preparation samples were quantified using fluorescence based method and RNA quality was assessed using RNA 6000 Nano Kit (Cat. 5067-1511, Agilent Technologies LDA UK Limited, Cheshire UK) on Bioanalyzer2100 (Agilent Technologies LDA UK Limited, Cheshire UK). 500 ng of total RNA was used for library preparation with RNA Integrity Numbers (RINs) varying from 5.7 to 10. Samples were processed following manufacturer's HS (High-Sample) instructions (part no. 15031048 Rev. E, Illumina, Chesterford UK) with 12 PCR cycles used to enrich RNA fragments. A subset of 12 final libraries was analysed using DNA 1000 Kit (Cat. 5067-1504, Agilent

Technologies LDA UK Limited, Cheshire UK) and average library length determined as 300 bp. All libraries were quantified using RT-qPCR. Serial dilutions were made in singlicate and 100,000x dilution used for quantification by KAPA Library Quantification Kit Illumina ROX Low (cat. KK4873, KAPA Biosystems, London UK). Libraries were normalised to 30 nM and pooled in equal volumes to create a balanced pool. The pool of libraries was quantified after serial dilutions in triplicate and 10,000x and 100,000x dilutions used for quantification. The pool was used for clustering on HiSeq4000 sequencing flow cell following the manufacturer's instructions at 200 pM concentration with 1% spike-in of PhiX control (Cat. FC-110-3001, Illumina, Chesterford UK). A total of three lanes of sequencing was performed using HiSeq4000 50 bp single-end run type for dual-indexed libraries. Demultiplexing was performed using bcl2fastq2 v.2.17 software (Illumina, Chesterford UK), allowing 0 mismatches.

Single-end 50-bp reads generated on the Illumina HiSeq sequencer were aligned to the human genome version GRCh38.p5 using TopHat v2.1.0 (www.ncbi.nlm.nih.gov/pubmed/19289445). Read counts were obtained using HTSeq-count v1.5.0-p1 (www-huber.embl.de/users/anders/HTSeq/doc/overview.html). Read counts were then normalized and tested for differential gene expression using the DESeq workflow v1.18.0 (https://www.ncbi.nlm.nih.gov/pubmed/20979621). Multiple testing correction was applied using the Benjamini-Hochberg method. Genes were selected as differentially expressed with a false discovery rate (FDR) <0.01.

Preparation of libraries for RNA-seq performed by the CRUK CI Genomics Core Facility, data analysis conducted by the CRUK CI Bioinformatics Core Facility.

2.3 Targeted protein analysis and Proteomics Techniques

2.3.1 Antibodies

The following antibodies were used for Western blotting and Rapid Immunoprecipitation Mass-spectrometry of Endogenous protein (RIME) [Mohammed et al., 2013]: ERβ1 PPG5/10 (MAI-81281) (Thermo Scientific Pierce, Rockford IL USA), Novocastra-ER-beta (EMR02-NCL-ER-BETA) (Leica Biosystems, Newcastle, UK), ERβ-antibody H150 (Sc8974) (Santa Cruz Biotechnology, Dallas TX, USA), GeneTex ERβ 70182 (Irvine, CA, USA), ERβ 06-629 (Merck Millipore, Watford, UK), Abcam 288 [14C8] (Cambridge, UK), AR (sc816) (Santa Cruz Biotechnology, Dallas TX, USA), FOXA1 (ab5089) (Abcam Cambridge, UK), E2F1 C20 (sc193), Mouse IgG (sc2025) (Santa Cruz Biotechnology, Dallas, Texas USA), Rabbit anti-beta actin (ab8227) (Abcam, Cambridge, UK), mouse anti-beta actin [AC-15] (ab6276) (Abcam Cambridge, UK).
Two non-commercially available mouse monoclonal antibodies, ERβ CWK-F12 (gift from Dr. B. Katzenellenbogen, University of Illinois, USA)[Choi et al., 2001] and ERβ MC10 (gift from Dr. J. Hawse, Mayo Clinic, Minnesota, USA)[Wu et al., 2012] were used. Details of the ERβ antibodies included in the validation study are summarised in table 2.2 and the locations of antibody binding on the ERβ protein shown in figure 2.1.

2.3.2 Protein extraction for Western blotting

Cell lines were harvested for nuclear protein extraction using the Ne-Per® nuclear extraction kit (Thermo Scientific Pierce, Rockford IL USA) according to the manufacturer's instructions. Extracted protein was quantified using the Direct Detect® system (Merrick Millipore, Massachusetts USA).

2.3.3 Western blotting

Nuclear protein extracts were prepared with 4X protein sample loading buffer (LI-COR Biosciences, USA), 10X NuPage sample reducing agent (Thermo Fisher

Antibody	Immunogen	Host species	Class	Binding region	Application
NCL-ER-BETA	Recombinant protein. Wt ERβ. C terminus	Mouse	Monoclonal	C terminus	IHC, WB
PPG5/10	Synthetic peptide C terminus of wt ERβ	Mouse	Monoclonal	C terminus	IF, IHC, WB
GeneTex 70182	Amino acids 1-153 of human ERβ expressed in E.coli	Mouse	Monoclonal	N terminus	IP, WB, ChIP
Millipore 06-629	Amino acids 46-63 of human ERβ	Rabbit	Polyclonal	NTD	WB, IHC
Santa cruz sc8974	Amino acids 1-150 of human ERβ	Rabbit	Polyclonal	N terminus	WB, ChIP, IF, ELISA
Abcam 288 [14C8]	Recombinant fusion protein. Amino acids 1-153 of human ERβ in E.coli	Mouse	Monoclonal	N terminus	WB, Flow cyt, IHC, ICC, ChIP
CWK-F12	Recombinant protein. Amino acids 256-505 of human wt ERβ	Mouse	Monoclonal	C-terminus	WB, IP, IHC
MC10	Fusion protein. Amino acids 1-140 of human ERβ in E.coli	Mouse	Monoclonal	N terminus	IHC, IP, WB, IF

Table 2.2: **Details of ERβ antibodies included in validation study,** according to manufacturers' published datasheets.

Scientific, Leicestershire, UK) and water, and 15 μg protein per lane loaded into Bolt 4-12% Bis-Tris gels (Thermofisher Scientific, Leicestershire, UK). Gels were run with MOPS running buffer for 30 minutes at 60V followed by 30 minutes at 120V.

Western transfer was performed using the iBlot system (Invitrogen, Paisley, UK) according to the manufacturer's instructions. Membranes were blocked with Odyssey® blocking buffer (LI-COR Biosciences, USA) for 1 hour at room temperature. Primary antibodies were added at the following dilutions in Odyssey® blocking buffer/PBS/0.1% tween and incubated overnight at 4°C: Novocastra 1:100, PPG5/10 1:100, CWK-F12 1:200, MC10 1:300, Sc8974 1:200, GeneTex 70182 1:200, Millipore 06-629 1:500 and Abcam 288 [14C8] 1:500, AR (sc816) 1:500, FOXA1 (ab5089) 1:1000. The following were used as loading controls according to the species of the primary antibody undergoing assessment: rabbit anti-beta actin 1:5000, mouse anti-beta actin 1:1000.

The membranes were washed 3 times each for 5 minutes with PBS/0.1% tween

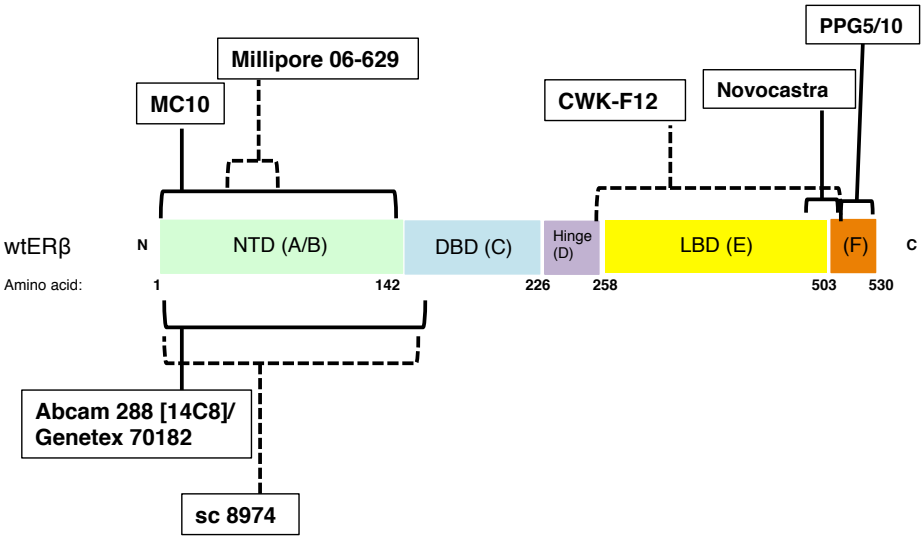


Figure 2.1: **Binding sites of ERβ antibodies included in validation study,** according to manufacturers' published datasheets.

and incubated with secondary antibodies in Odyssey® blocking buffer/PBS/0.1% tween for 1 hour at room temperature: Goat anti-mouse (green) 1:5000, Goat anti-rabbit (red) 1:20000. Membranes were imaged to visualise the bands using the Li-Cor Odyssey® fluorescent imaging system (LI-COR Biosciences, USA) at 169 μm wavelength, medium quality, intensity rating 5 as per provided instructions.

2.3.4 Rapid Immunoprecipitation Mass-spectrometry of Endogenous proteins (RIME)

RIME experiments were conducted as previously described [Mohammed et al., 2013]. Briefly, cells were grown in 15 cm^2 plates in appropriate culture media. For antibody validation experiments in the MDA-MB-231-ERβ cell line, 4 x 10^7 cells were used for each experimental condition. For all other RIME experiments, 8 x 10^7 cells were used per condition.

Cells were crosslinked with media containing 1% EM grade formaldehyde (TEBU biosciences) for 8 minutes and the formaldehyde then quenched with 0.1M glycine. Cells were washed, harvested and pelleted in cold PBS. The nuclear fraction was

enriched by resuspending the cell pellet in 10 ml of lysis buffer 1 (LB1) (50 mM HEPES-KOH [pH 7.5], 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP-40 or Igepal CA-630, and 0.25% Triton X-100) for 10 minutes at 4°C. Cells were then pelleted and resuspended in LB2 (10 mM Tris-HCL [pH 8.0], 200 mM NaCl, 1 mM EDTA, and 0.5 mM EGTA) for 5 minutes at 4°C. Cells were pelleted and resuspended in 300 μl of LB3 (10 mM Tris-HCl [pH 8], 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.1% Na-deoxycholate, and 0.5% N-lauroylsarcosine) and sonicated in a waterbath sonicator (Diagenode Bioruptor®, Diagenode, Seraing, Belgium) for 45 minutes. A 10 μl aliquot of sonicated chromatin was reverse cross-linked at 95°C for 10 minutes and run on a 2% E-Gel (Thermo Fisher Scientific, Leicestershire, UK) to confirm sufficient sonication and that fragments were 200-300 base pairs in length. 30 μl of 10% Triton-X was added and the sonicated lysate centrifuged at 17,000G for 10 minutes to remove cell debris.
The supernatant was then incubated with 100 μl of magnetic beads (Protein G Dynabeads®, Dynal), which had been previously washed three times with PBS containing 5 mg/ml BSA, incubated with the appropriate volume of antibody overnight rotating at 4°C overnight and then washed three times with PBS containing 5 mg/ml BSA. The washed beads and sonicated samples were incubated overnight at 4°C. Beads were collected using a magnetic concentrator, the supernatant removed and the beads washed 10 times in 1 ml ice-cold RIPA buffer (50mM HEPES pH 7.6, 1mM EDTA, 0.7% Na deoxycholate, 1% NP-40, 0.5M LiCL) and twice in 100 mM ammonium hydrogen carbonate (AMBIC) solution. Dry, frozen beads were submitted for tryptic digestion of bead-bound protein, and peptides pulled down by immunoprecipitation (IP) identified by mass-spectrometry (LTQ Velos-Orbitrap MS).

RIME data analysis

Raw MS data files were processed using Proteome Discoverer v.1.3 (Thermo Scientific). Processed files were searched against the SwissProt human database using the Mascot search engine version 2.3.0 with a FDR of <1%. For each ChIP, the resulting list of purified peptides identified by MS was filtered against the corresponding IgG negative control to remove non-specific proteins pulled

down. Mean percentage peptide coverage, and mean number of unique peptides identified in biological duplicate experiments were calculated.

2.3.5 Parallel Reaction Monitoring (PRM)

With Dr. A. Groen, Proteomics Core Facility, CRUK Cambridge Institute.

Nuclear pellets from cell lines were prepared using the Panomics nuclear extraction kit (Affymetrix, CA USA) as per the manufacturer's provided instructions. Nuclear pellets were lysed in 8M Urea, 0.1% SDS in 50 mM TEAB by waterbath sonication for 2 x 5 minutes. After protein estimation 20 μg of protein was taken for tryptic digestion. 50 mM of TEAB (pH = 8) was added up to a total volume of 100 μl. Cysteines were reduced in 0.1 mM DTT for 1 hour at room temperature and alkylated in 0.1 mM IAA for 30 minutes at room temperature in the dark. Alkylation was quenched by adding 0.1 mM DTT for 15 minutes. Trypsin (Promega trypsin (V5111)) was added in a 1:100 trypsin:protein ratio for 1 hour at room temperature. Another batch of trypsin (1:100 ratio) was added to have a final ratio of 1:50 for incubation overnight. Next day samples were acidified to a final concentration of 1% formic acid and subsequently cleaned over C18 spin columns (Harvard apparatus C18 Micro SpinColumn^{TM}). Finally after elution from the columns samples were lyophilised in a speedvac and resolubilised in 0.1% formic acid, 5% ACN to a final peptide concentration of 1 μg/μl. Samples were subjected to liquid chromatography-electrospray ionization in an Orbitrap nano-ESI Q-Exactive mass spectrometer (Thermo Scientific, Leicestershire, UK), coupled to a nanoLC (Dionex Ultimate 3000 UHPLC). Samples were trapped on a 100 μm 2 cm, C18, 5 μm, 100 trapping column (Acclaim PepMap 100) in μL-pickup injection mode at 4 μL/min flow rate for 10 minutes. Samples were then loaded on a Rapid Separation Liquid Chromatography, 75 μm 25 cm nanoViper C18 3 μm 100 column (Acclaim, PepMap) retrofitted to an EASY-Spray source with a flow rate of 300 nL/min (buffer A, HPLC H_2O, 0.1% formic acid; buffer B, 100% ACN, 0.1% formic acid; 60-min gradient; 0-5 min: 5% buffer B, 5-45 min: 5 to >56% buffer B, 45.1 to 50 min: 56% to >95% buffer B, 50.1 to 60 min, 5% buffer B). Peptides were transferred to the gaseous phase with positive

ion electrospray ionization at 1.8 kV. Precursors were targeted in a 2Th selection window around the m/z of interest. Precursors were fragmented in high-energy collisional dissociation mode with normalised collision energy dependent on the target peptide. The first mass analysis was performed at a 70,000 resolution, an automatic gain control target of 3 x 10^6, and a maximum C-trap fill time of 200 milliseconds; MS/MS was performed at 35,000 resolution, an AGC target of 5 x 10^4, and a maximum C-trap fill time of 100 milliseconds. Spectra were analysed using Skyline with manual validation.

2.4 Immunohistochemistry (IHC)

2.4.1 Preparation of MDA-MB-231-ERβ cell pellets

Formalin-fixed, paraffin-embedded MDA-MB-231-ERβ- and MDA-MB231-ERβ+ cell pellets were generated for IHC validation of the CWK-F12 ERβ antibody, with $\sim$2 x 10^7 cells per pellet. ERβ expression was induced by addition of 0.5 μg/ml doxycycline for 24 hours. Antigen retrieval was achieved by incubating in citrate-based retrieval solution for 20 minutes. Sections were stained using CWK-F12 ERβ antibody, diluted 1:250 in standard Bond diluent using Leica's Polymer Refine Kit (Catalogue No: DS9800) on the automated Bond platform (Leica Biosystems Newcastle Ltd, Newcastle UK). Images were captured using Aperio® software (Leica Biosystems Newcastle Lt, Newcastle UK).

2.4.2 Prostate tissue microarrays

The mini-prostate tissue microarray (TMA) was created from a random selection of prostate cancers, including a range of different tumour grades, and benign prostatic tissue (10 cancer, 5 benign in total) (ethical approval: ProMPT study MREC/01/4/061). The areas to be sampled from the formalin-fixed and paraffin embedded tissue donor blocks were marked on the original corresponding haematoxylin and eosin-stained paraffin sections. Each donor block was assessed to ensure that there was an adequate amount of tissue available for sampling, and cores of tissue were punched from the selected area of the donor block using 5

mm skin biopsy punches. Each core was then re-embedded into a new recipient paraffin block and its position in the block recorded on a TMA map. Cores of pig kidney were used as markers for orientation purposes.

The degarelix TMA has been previously published [Shaw et al., 2016]. It was constructed using prostate tissue obtained by radical prostatectomy taken from 27 men with high-risk prostate cancer, treated preoperatively with 240 mg degarelix, and 20 untreated control patients. ERβ expression from areas of cancer and benign tissue in both treated and untreated patients were scored in a blinded fashion by Dr. A.Y. Warren (Consultant Histopathologist, Cambridge University Hospitals NHS Foundation Trust) using the Allred scoring system [Harvey et al., 1999] (ethical approval: REC ref:11/H0311/2 and REC ref:01/4/061).

In both the mini-prostate and degarelix TMA, 3.5 μm sections were cut from the recipient paraffin block, and mounted onto charged slides which, when dried, were sealed with paraffin wax. The CWK-F12 ERβ antibody was optimised to the clinical samples and diluted at 1:200 in antibody diluent consisting of 1% donkey serum, 0.05% Tween20 in 300 mM TBS to reduce background staining. Antigen retrieval was achieved by incubating in Tris EDTA for 20 minutes at 100°C. Images were captured at 250x magnification using Image Pro-Insight (Media Cybernetics. Rockville, MD. USA).

2.5 Chromatin Immunoprecipitation and high throughput sequencing (ChIP-seq)

ChIP-seq was performed according to a previously published protocol [Schmidt et al., 2009], with some alterations detailed below.

2.5.1 Crosslinking, lysis and sonication

Four 15 cm^2 plates were used per condition. Cells were crosslinked in Solution A (0.1 M NaCl, 1.0 mM EDTA, 0.5 mM EGTA, 50 mM HEPES) containing 1% formaldehyde for 10 minutes and the formaldehyde then quenched with 0.1M

glycine. Cells were washed, harvested and pelleted in cold PBS. The nuclear fraction was enriched by resuspending the cell pellet in 10 ml of LB1 for 10 minutes at 4°C. Cells were then pelleted and resuspended in LB2 for 5 minutes at 4°C. Cells were pelleted and resuspended in 300 μl of LB3 and sonicated in the Bioruptor® for 30 minutes. A 10 μl aliquot of sonicated chromatin was reverse cross-linked at 95°C for 10 minutes and run on a 2% E-Gel (Thermo Fisher Scientific, Leicestershire, UK) to confirm sufficient sonication and that fragments were 200-300 base pairs in length. 30 μl of 10% Triton-X was added and the sonicated lysate centrifuged at 20,000G for 10 minutes to remove cell debris.

2.5.2 Preparation of magnetic beads and immunoprecipitation

Fifty microlitres of lysate was removed and stored at -20°C to be used as input. The remaining supernatant was then incubated with 100 μl of magnetic beads (Protein G Dynabeads®, Dynal), which had been previously washed three times with PBS containing 5 mg/ml BSA, incubated with the appropriate volume of antibody overnight rotating at 4°C overnight and then washed three times with PBS containing 5 mg/ml BSA. The washed beads and sonicated samples were incubated overnight at 4°C. Beads were collected using a magnetic concentrator, the supernatant removed and the beads washed 6 times in 1 ml ice-cold RIPA buffer. Beads were washed once in 1 ml TE. 200 μl of elution buffer (50 mM TrisHCl, pH8, 10 mM EDTA, 1% SDS) was added and the beads vortexed. 150 μl elution buffer was added to the input sample. Both ChIP and input samples were incubated in a water bath at 65°C overnight to reverse cross-link DNA.
Following incubation and using a magnetic rack, the supernatant was transferred to a fresh Eppendorf and 200 μl TE added. RNAse A (8 μl of 1 mg/ml) was added and the samples incubated at 37°C for 30 minutes. Proteinase K (4 μl of 20 mg/ml) was added and the samples incubated at 55°C for 1 hour. Phenol:chloroform:isoamyl alcohol cleanup was performed using pre-spun Eppendorf phase-lock columns. Following centrifugation for 5 minutes at 10,000 G the upper layer was removed and added to 16 μl of 5M NaCl with 2 μl glycogen (20 μg/μl). 800 μl of 80% ethanol was added and samples left at -80°C for 1 hour. Samples

were centrifuged at 21,000 G for 20 minutes.
The supernatant was removed and the pellet washed in 70% ethanol, then left to air dry at 37°C for <10 minutes. Pellets of ChIP and input samples were resuspended in 13 μl or 20 μl 10 mM Tris HCl pH 8.0 respectively and stored at -20°C.

2.5.3 Library preparation and Illumina Sequencing

DNA fragment length of input samples was measured using the Agilent 2100 Bioanalyzer system with Agilent DNA 1000 Kit (Agilent Technologies, Santa Clara, CA, USA). The concentration of DNA in input samples was quantified using the Quant-it^{TM} dsDNA Assay Kit (broad range) and the PHERAstar® (Thermo Fisher Scientific, Leicestershire, UK). Input samples were diluted in 10 nM Tris HCl to a concentration of 0.5 ng/μl.
Library preparation was carried out using the ThruPLEX® DNA-seq Kit (Cat. no. R400407, Rubicon Genomics, Ann Arbor, MI, USA) according to the manufacturer's provided instructions. Twelve cycles were used in the amplification step. Agencourt AMPure XP beads (Beckman Coulter Life Sciences, Indianapolis, USA) were used to purify the DNA following the manufacturer's provided protocol. DNA fragment lengths of all samples (ChIPs and inputs) were assessed using the Bioanalyzer and samples quantified using the Kapa Library Quantification kit (Kapabiosystems, London, UK).
Libraries were pooled at equal concentration and submitted for one lane of sequencing on the HiSeq 2500 (Illumina Inc. San Diego, CA, USA) to check the balance of indexes in the submitted pool. These data were then used to re-balance the pool according to the relative representation of each index. The re-balanced pool was then sequenced to generate a total of 2 x 10^7 reads per sample.

2.5.4 Analysis of ChIP-seq data

Performed by Dr. Igor Cherneukhin, Carroll group, CRUK Cambridge Institute

Sequenced reads were mapped to hg38 genome using bowtie2 2.2.6 [Langmead and Salzberg, 2012]. Aligned reads with the mapping quality less than 5 were

filtered out. The read alignments from four replicates were combined into single library and peaks were called with MACS2 version 2.0.10.20131216 [Zhang et al., 2008] using sequences from LNCaP-ERβ cells' chromatin extracts as a background input control. The peaks yielded with MACS2 q value $\leq$ 1 x 10^4 were selected for downstream analysis. Meme version 4.9.1 [Bailey et al., 2009] was used to detect known and discover novel binding motifs amongst tag-enriched sequences. For visualizing tag density and signal distribution heatmap the read coverage in a window of +/- 2.5 or 5 kb region flanking the tag midpoint was generated using the bin size of 1/100 of the window length. Differential binding analysis (Diffbind) was performed as described previously (Stark, R., Brown, G. D. 2011. DiffBind: differential binding analysis of ChIP-Seq peak data. Bioconductor http://bioconductor.org/packages/release/bioc/html/DiffBind.html).

To integrate the ChIP-seq and RNA-seq datasets, ChIP-seq binding sites were annotated using genomic features of the Human Genome Assembly (GCR-h38). Genes whose coordinates were mapped in the proximity of +/- 50kb to ChIP-seq peak summits were selected as potential target genes for regulation by the studied factor. RNA-seq expression values of the potential target genes were extracted and normalised using library size coefficiencies and standard deviation values. Bioinformatics toolbox of Matlab framework (https://uk.mathworks.com) was used as a source for hierarchical clustering functions and also heatmap visualisation.

2.6 Statistical analyses

2.6.1 Analysis of cell proliferation assay (Section 2.1.5)

To assess differences between treatment conditions in the IncuCyte^{TM} experiment, cell confluence was normalised to the starting confluence in each well. Technical replicates were averaged to give a mean result per biological replicate. Biological replicates were averaged to give a mean result per treatment condition. Final percentage change in cell confluence at 7 days was compared between conditions using unpaired t-tests performed in GraphPad Prism version 6 to determine sta-

tistical significance. Error bars indicate standard deviation and a p value of <0.05 was considered statistically significant.

2.6.2 Analysis of mRNA expression data (Section 2.2.3)

mRNA expression data as determined by RT-qPCR were analysed using unpaired t-tests in GraphPad Prism version 6. Differences were considered statistically significant at a p value of <0.05. Data presented are mean of technical triplicate experiments +/- standard deviation.

2.6.3 Analysis of ERβ expression in degarelix TMA (Section 2.4.2)

Statistical analysis by Dr. Matthew Eldridge, CRUK Cambridge Institute.

Allred scores from the ERβ-stained degarelix TMA were compared using a Wilcoxon rank sum test with continuity correction as previously described [Shaw et al., 2016].

2.6.4 Interrogation of clinical datasets (Section 5.2.1)

The CamCap dataset [Ross-Adams et al., 2015] was interrogated using a published, online software tool [Dunning et al., 2017]. This dataset was generated by integration of copy number analysis and transcriptomics data from 482 tumour, benign and germline samples obtained from 259 men with primary prostate cancer. The integrated data were then used to identify five prognostic groups with distinct transcript expression profiles and genomic alterations (iClusters). Variability in gene expression across the iClusters was assessed by analysis of variance (ANOVA).

Data from two published datasets [Rajan et al., 2014; Shaw et al., 2016] comparing gene expression in prostate tissue pre- and post-treatment with ADT were interrogated for ERβ mRNA expression (GSE48403 and GSE72920). Expression

was analysed by unpaired t-tests in GraphPad Prism version 6, with statistical significance set at <0.05.

Chapter 3

Validation of Estrogen Receptor Beta Antibodies

3.1 Introduction

3.1.1 Essential background information

The role of ERβ in both physiological and disease states remains uncertain [Bottner et al., 2014; Haldosen et al., 2014; Nelson et al., 2014]. Despite a large body of published literature regarding ERβ in prostate cancer, there is still controversy as to whether its predominant role is tumour-suppressive [Attia and Ederveen, 2012; Chang and Prins, 1999; Ellem and Risbridger, 2007; Horvath et al., 2001; McPherson et al., 2010; Muthusamy et al., 2011; Nakajima et al., 2011; Zhu et al., 2004] or tumour promoting [Yang et al., 2012, 2015; Zellweger et al., 2013]. Much of the evidence concerning the hypothesised role of ERβ arises from ERβ expression profiles in prostate tissue as determined by IHC [Asgari and Morakabati, 2011; Attia and Ederveen, 2012; Bouchal et al., 2011; Dey et al., 2014; Horvath et al., 2001; Leav et al., 2001; Risbridger et al., 2007; Zellweger et al., 2013; Zhu et al., 2004]. There are, however inconsistencies in the findings between these studies, contributing to the uncertainty regarding the role of ERβ in cancer.

In prostate cancer, previous studies showed that ERβ is highly expressed in benign luminal epithelial cells, with expression declining in cancer development.

ERβ expression therefore inversely correlated with increasing Gleason grade of cancer [Asgari and Morakabati, 2011; Attia and Ederveen, 2012; Dey et al., 2014; Horvath et al., 2001; Leav et al., 2001; Risbridger et al., 2007]. However, it has also been reported that ERβ expression is high in bone and lymph node metastases [Bouchal et al., 2011; Zhu et al., 2004], and that high ERβ expression correlates with poor clinical prognosis [Horvath et al., 2001; Zellweger et al., 2013]. One potential biological explanation for this is the expression of ERβ isoforms, which are fully conserved in exons 1 - 6, but have different C-terminal domains [Leung et al., 2006a] (see section 1.2.2 for details). Different antibodies may have higher affinity for either the conserved N-terminal region or the variant C-terminal end of the protein. This may, therefore give different IHC results in prostate cancer, where it has been reported that ERβ isoform 2 (ERβ2) expression increases with advanced and castrate-resistant disease [Dey et al., 2012; Leung et al., 2010].

However, it is well documented in the literature that antibodies to ERβ are problematic, with marked variation in specificity and sensitivity to ERβ [Choi et al., 2001; Hartman et al., 2012; Skliris et al., 2002; Weitsman et al., 2006]. This results in discrepancy and controversy surrounding the expression profile of ERβ in tissues and its molecular role as determined by antibody-dependent functional studies. This is likely to have contributed to the conflicting conclusions regarding ERβ.

A number of ERβ antibody validation studies have been previously published. Choi *et al.* [Choi et al., 2001] generated three monoclonal ERβ antibodies and assessed them by Western blotting using baculovirus-mediated expression of ERβ protein. These antibodies were further assessed in rat ovary and epididymis by IHC, where tissue-specific expression of ERβ was found to correlate with mRNA expression [Hess et al., 1997; Rosenfeld et al., 1999]. Furthermore, Skliris *et al.* evaluated seven ERβ antibodies by Western blotting and IHC of benign breast tissue, including two antibodies (PPG5/10 and 14C8) assessed in the current study. Lastly, Wu *et al.* [Wu et al., 2012] developed a monoclonal ERβ antibody MC10 (included in the present study) and tested it by Western blotting in a U2OS cell line with inducible ERβ expression and by IHC in breast tissue.

Despite these attempts to address the problems associated with ERβ antibodies, many researchers have continued to work on ERβ biology using non-validated antibodies, or antibodies that are only validated for one particular experimental technique. It has been shown that not all antibodies are specific to their target across multiple experimental platforms [Baker, 2015; Bordeaux et al., 2010], indicating that this is not a reasonable assumption to make.

A limitation of some previous antibody validation studies is that they rely on two key assumptions; namely that when assessing an antibody by Western blotting in a cell line model, the factor of interest is expressed and secondly, when assessing an antibody's specificity by IHC in tissue, the tissue expression of the factor has been well characterised. In the case of ERβ these assumptions are problematic, as its expression in commonly used cell line models [Al-Bader et al., 2011; Holbeck et al., 2010; Nakajima et al., 2011; Shaaban et al., 2003; Skliris et al., 2002; Zhou et al., 2012] and in tissues [Asgari and Morakabati, 2011; Attia and Ederveen, 2012; Bouchal et al., 2011; Dey et al., 2014; Gruvberger-Saal et al., 2007; Guo et al., 2014a,b; Hieken et al., 2015; Horvath et al., 2001; Leav et al., 2001; Omoto et al., 2002; Risbridger et al., 2007; Umekita et al., 2006; Zellweger et al., 2013; Zhu et al., 2004] is not universally accepted. As such, previous assumptions of ERβ expression in cell line models and cancer tissue might hinder genuine validation of reagents.

3.1.2 Aims

The first priority of this study therefore was to test and validate a panel of ERβ antibodies, in a manner that addresses the aforementioned assumptions. Commercially available and non-commercially available antibodies obtained from collaborators [Choi et al., 2001; Wu et al., 2012] were assessed by multiple independent techniques. In a cancer cell line model with stable, doxycycline-inducible ERβ expression (MDA-MB-231-ERβ), antibodies were firstly assessed for protein detection by Western blotting. Secondly, antibodies were assessed by Rapid Immunoprecipitation Mass spectrometry of Endogenous protein (RIME) [Mohammed et al., 2013]. Thirdly, the CWK-F12 ERβ antibody validated using the

above techniques was subsequently optimised for IHC and validated using the inducible MDA-MB-231-ERβ cell line model as positive and negative controls. ERβ antibody validation has not previously been explored in such a systematic manner.

3.1.3 Key Findings

1. The MDA-MB-231-ERβ cell line serves as a valid model for ERβ antibody validation, demonstrating strong induction of ERβ mRNA and protein expression with doxycycline treatment and no ERβ mRNA or protein expression in the absence of doxycycline.

2. Assessment of the eight ERβ antibodies by Western blotting demonstrated high variability in antibody specificity with one of the most commonly published antibodies (NCL-ER-BETA) yielding a false-positive result.

3. RIME analysis confirmed that the NCL-ER-BETA antibody is not specific to ERβ. Other antibodies were shown to have variable levels of protein coverage and specificity.

4. The results demonstrate that two of the antibodies evaluated (MC10 and CWK-F12) are specific to ERβ across multiple experimental platforms.

3.2 Results

3.2.1 Validation of the MDA-MB-231-ERβ cell line model

The MDA-MB-231-ERβ cell line was provided by the laboratory of Dr. J. Hawse (Mayo Clinic, Minnesota USA) [Reese et al., 2014]. MDA-MB-231 is a 'triple negative' breast cancer cell line model into which the Hawse laboratory stably transfected doxycycline-inducible ERβ expression using the T-Rex™system (Invitrogen, Fisher Scientific UK, Loughborough, UK). Importantly, following transfection of the tetracycline-repressor (TET-R) construct, clonal selection was used, followed by assessment of the TET-R activity by transient transfection with a *luciferase* reporter. The advantage of this approach is that the uninduced 'off' condition is known to be robust, with minimal 'leakiness' of expression from the promotor, thus providing an ideal model for antibody validation.

The workflow of the antibody validation process is described in figure 3.1. MDA-MB-231-ERβ cells were treated for 24 hours with doxycycline to induce ERβ expression. Expression of ERβ mRNA was confirmed by RT-qPCR and shown to be dose-dependent (Figure 3.2). Treatment with 0.1 μg/ml doxycycline induced approximately 70-fold expression of ERβ and 0.5 μg/ml doxycycline resulted in approximately 120-fold induction of expression ($p = 0.01$ and $p = 0.003$ respectively).

3.2.2 Assessment of ERβ antibodies by Western blotting

A panel of six of the most commonly used, commercially available ERβ antibodies were selected for evaluation [Al-Bader et al., 2011; Asgari and Morakabati, 2011; Bouchal et al., 2011; Celhay et al., 2010; Chen et al., 2009; Ciucci et al., 2014; Ellem et al., 2014; Foryst-Ludwig et al., 2008; Grubisha et al., 2012; Han et al., 2015; Hussain et al., 2012; Madak-Erdogan et al., 2013; Mak et al., 2013, 2015a; McPherson et al., 2007, 2010; Nakajima et al., 2011; Oliveira et al., 2007; Rossi et al., 2011; Shaaban et al., 2003; Umekita et al., 2006; Vivar et al., 2010; Yang et al., 2012, 2015; Zellweger et al., 2013; Zhou et al., 2012], along with two non-commercially available antibodies obtained from collaborators (MC10 from Dr.

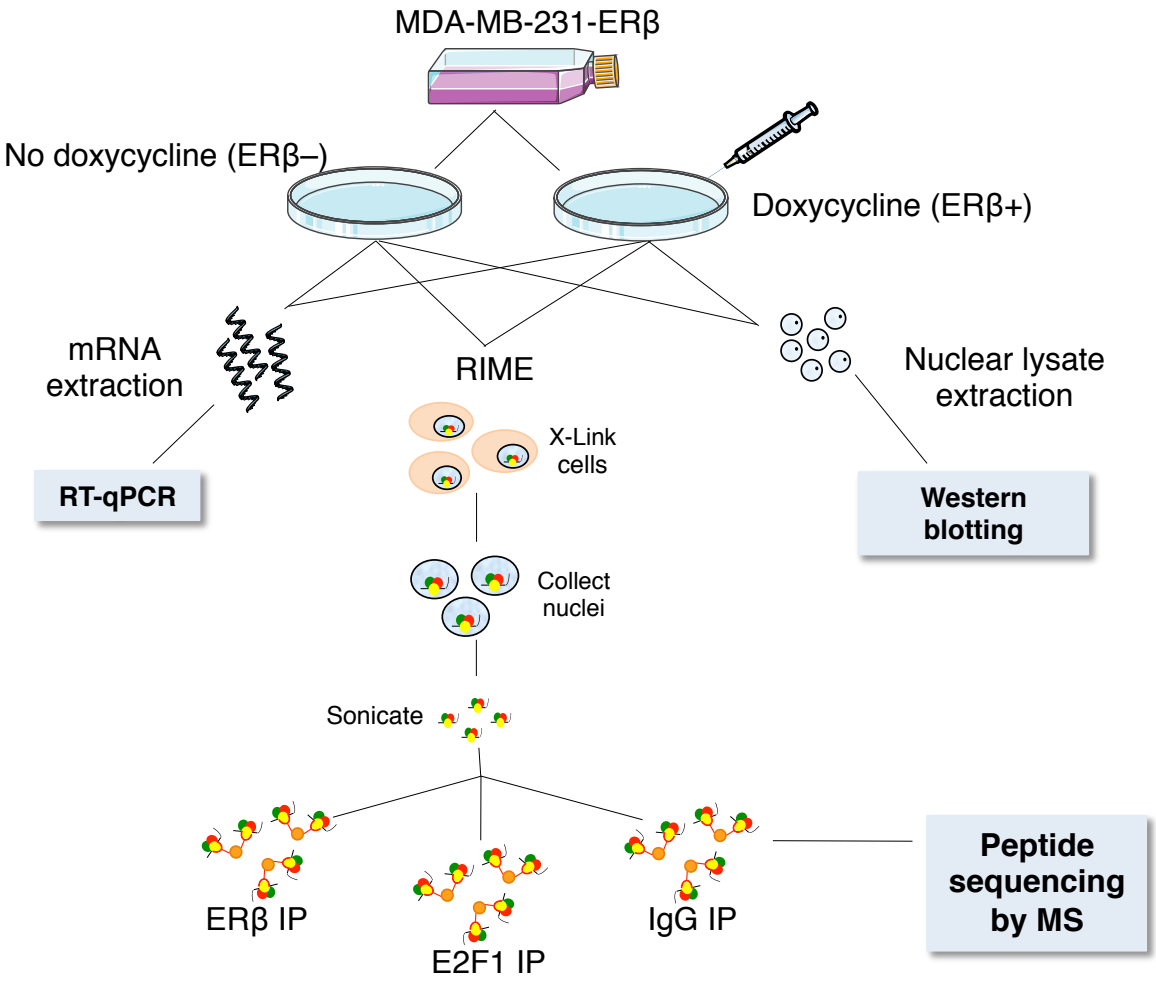


Figure 3.1: **Experimental workflow of ERβ antibody validation**. Doxycycline-induced expression of ERβ was confirmed by RT-qPCR. Nuclear lysate was collected for Western blotting of ERβ protein. MDA-MB-231-ERβ- and MDA-MB-231-ERβ+ cells were crosslinked and immunoprecipitated with antibody coupled with beads, and mass spectrometry (MS) performed to identify pulled-down peptides.

John Hawse, Mayo Clinic, Minnesota USA [Wu et al., 2012] and CWK-F12 from Prof. Benita Katzenellenbogen, University of Illinois, Illinois USA [Choi et al., 2001]) (Table 3.1).

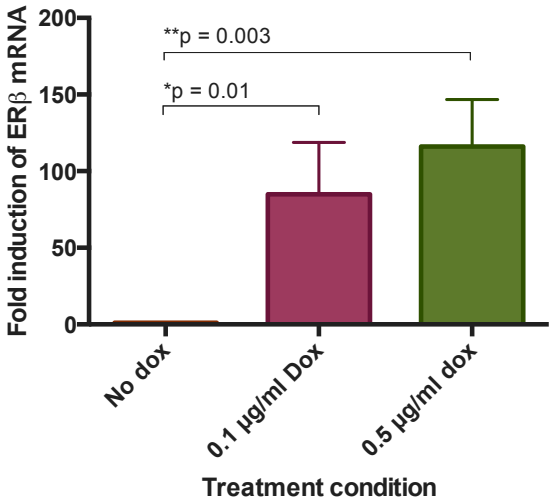


Figure 3.2: **Induction of ERβ expression in doxycycline-treated MDA-MB-231-ERβ cells**. MDA-MB-231-ERβ cells were treated with 0.1 µg/ml or 0.5 µg/ml doxycycline for 24 hours. Untreated MDA-MB-231-ERβ- cells provided a negative control. Data are mean + S.D. of technical triplicate experiments.

Western blots of MDA-MB-231-ERβ+ and MDA-MB-231-ERβ- nuclear lysates were performed using above mentioned antibodies (Figure 3.3). The PPG5/10 antibody detected a protein band of 77 kDa, but importantly there was no difference between ERβ+ or ERβ- conditions, suggesting that it recognises a non-specific protein. Similarly, the NCL-ER-BETA antibody detected a band of ∼59 kDa, which is the correct size for ERβ, however there was no difference between ERβ+ or ERβ- conditions, implying that this band was not ERβ. The GeneTex 70182 antibody detected a band of 59 kDa with differential signal between ERβ+ and ERβ- conditions, with an additional non-specific band present at around 65 kDa. The Millipore 06-629 antibody detected a band of 59 kDa in both ERβ+ and ERβ- conditions. This band was stronger however, in the ERβ+ condition, suggesting that the antibody could be cross-reacting with another protein of 59 kDa in addition to detecting ERβ. Our RIME data suggests that this may be cross-reactivity with LACTB (discussed in section 3.2.3). MC10, CWK-F12, Abcam 288 [14C8] and sc8974 ERβ antibodies all detected protein bands of 59 kDa

ERβ Antibody	NCL-ER-BETA	Genetex 70182	PPG5/10	Abcam 288 [14C8]	Millipore 06-629	Santa Cruz 8974
Reference	Ellem 2014	Celhay 2010	Asgari 2011	Abd-Elmageed 2013	Bouchal 2011	Al Bader 2011
	Hussain 2012	Madak-Erdogan 2013	Ciucci 2014	Colciago 2014	Chen 2009	Foryst-Ludwig 2008
	McPherson 2007	Mak 2013	Shaaban 2003	Cotrim 2013	Grubisha 2012	Han 2015
	McPherson 2010	Mak 2015	Wimberly 2014	Dey 2012		Rossi 2011
	Oliveira 2007	Nakajima 2011		Dey 2014		Zhou 2012
	Yang 2015			Setlur 2008		
	Zellweger 2013			Shaaban 2003		
	Umekita 2006			Vivar 2010		
				Yang 2012		

Table 3.1: **Commonly used ERβ antibodies in published literature**. Six of the most commonly used, commercially available ERβ antibodies were chosen for validation, listed here with papers relevant to breast and prostate cancer in which they were published.

with differential signal between ERβ+ and ERβ- conditions, confirming their specificity to ERβ by Western blotting.

3.2.3 Assessment of ERβ antibodies by RIME

The eight ERβ antibodies were then assessed by RIME. This uses chromatin immunoprecipitation (ChIP) followed by mass spectrometry (MS) to identify enriched peptides [Mohammed et al., 2013]. MDA-MB-231-ERβ cells were treated for 24 hours with 0.5 μg/ml doxycycline to induce maximal ERβ protein expression (Figure 3.2) and untreated MDA-MB-231-ERβ- cells served as a negative control. In both conditions, RIME purification of E2F1 was included in parallel as a positive control, since E2F1 is a ubiquitously expressed protein and the

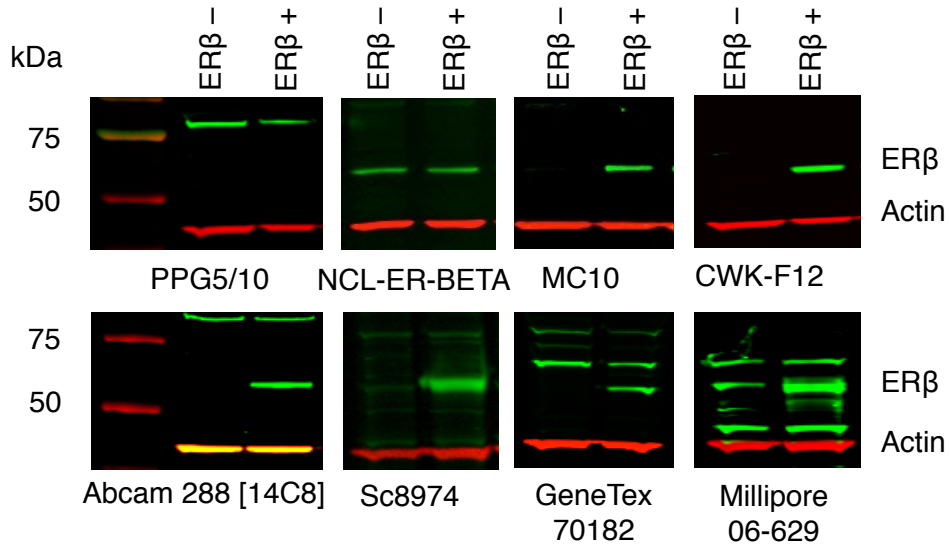


Figure 3.3: **Validation of ERβ antibodies by Western blotting in MDA-MB-231-ERβ+ and MDA-MB-231-ERβ- nuclear lysates**. PPG5/10 antibody did not detect a band of appropriate size to be ERβ. NCL-ER-BETA detected bands of appropriate size to be ERβ with no difference between ERβ+ and ERβ- conditions. MC10, CWK-F12, Abcam 288 [14C8] and sc8974 antibodies detected bands of ∼59 kDa, with differential signal between ERβ+ and ERβ- conditions indicating specificity to ERβ. GeneTex 70182 detected ERβ, with non-specific signal at 65 kDa. Millipore 06-629 appears to detect ERβ, although there is a 59 kDa band in the ERβ- condition.

antibody has been validated as being specific to E2F1 (Figure 3.4A). An IgG antibody specific to the species of the ERβ antibody was used to identify non-specific peptides pulled out by the IP. Any peptides pulled down in either replicate of the IgG were subtracted from the list of peptides identified by the ERβ RIME to generate a list of peptides specific to the ERβ IP for each antibody. Importantly, ERβ was not identified in any of the IgG RIMEs, and furthermore ERβ was not identified by any antibody in the ERβ- condition, confirming no expression of ERβ in the uninduced MDA-MB-231-ERβ cells (Figure 3.4C). In MDA-MB-231-ERβ+ cells, RIME revealed diverse peptide coverage by the different antibodies (Figure 3.4B). The most striking finding was that the NCL-ER-BETA antibody did not identify any ERβ peptides by RIME, confirming its lack of specificity for ERβ.

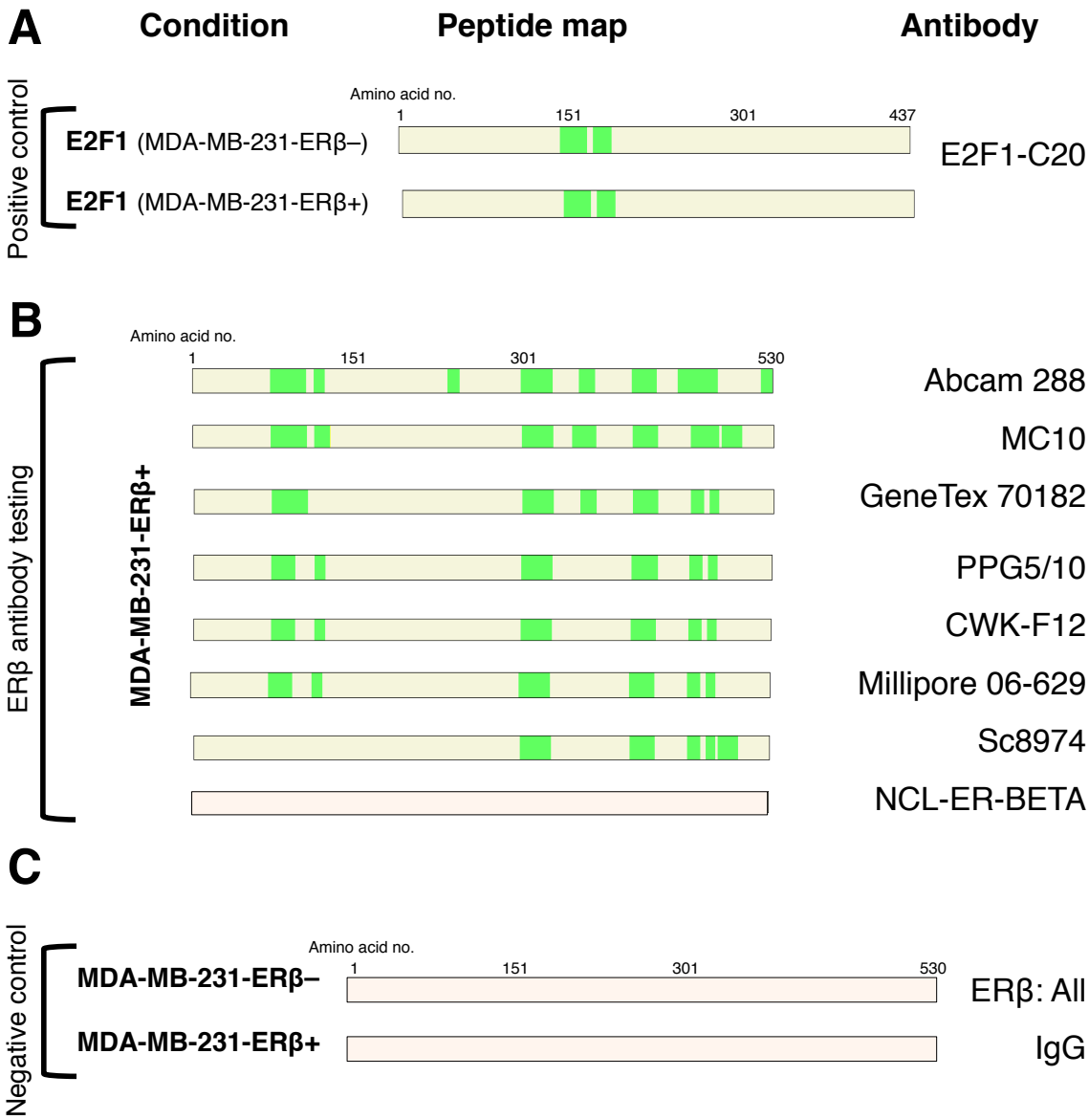


Figure 3.4: **Validation of ERβ antibodies by RIME reveals diverse peptide coverage**. (A) E2F1 positive control in ERβ+ or ERβ- conditions. (B) ERβ antibody tests in MDA-MB-231-ERβ+ cells revealed diverse peptide coverage by each antibody. NCL-ER-BETA did not identify any ERβ peptides. (C) Negative controls: All ERβ antibodies were tested in MDA-MB-231-ERβ- cells, to confirm absence of ERβ expression. None of the IgG antibodies purified ERβ.

A number of factors were considered to facilitate comparison of the ERβ antibodies (Table 3.2). Firstly the coverage of the protein, which indicates the percentage of the full-length protein identified by MS with a false discovery rate of ≤1%. Secondly, the mean number of unique ERβ peptides (mean of two independent biological replicates) was calculated for each ERβ antibody. Finally, to provide an indication of the specificity of each antibody, all the proteins pulled down by the IP and identified by MS were ranked according to the mean number of unique peptides. We hypothesised that the higher the ranking of ERβ, the greater the specificity of the antibody. Hence, if ERβ has the greatest number of unique peptides relative to all other proteins identified in the RIME, it is ranked 1^{st}.

Antibody	Coverage (%)	Mean no. Unique Peptides	No. Interacting proteins	ERβ Rank
Abcam 288	31.9	14.5	285	20
MC10	28.2	11.5	75	1
GeneTex 70182	26.1	11.5	11	1
PPG5/10	25.0	11.0	12	3
CWK-F12	17.7	7	64	2
Millipore 06-629	16.5	6.5	14	3
Sc8974	12.0	4.5	2	1
NCL-ER-BETA	0	0	0	n/a

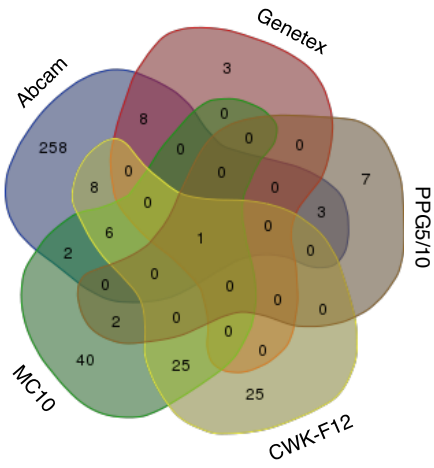
Table 3.2: **Comparison of ERβ antibody characteristics by RIME**. Summary of the characteristics of each ERβ antibody assessed by RIME, showing peptide coverage, mean number of unique ERβ peptides identified by MS, the number of other interacting proteins also pulled down in the IP and the ERβ ranking, used as an indicator of antibody specificity.

NCL-ER-BETA did not pull-down any ERβ peptides (Figure 3.4B), which is consistent with the lack of specificity by Western blotting (Figure 3.3). The Millipore 06-629 antibody pulled down ERβ in the test condition, although coverage and ranking were not as favourable as compared with some of the other antibodies. Interestingly, LACTB, a 60 kDa protein was pulled down by Millipore 06-629 in

both ERβ+ and ERβ- conditions, which may explain the ∼60 kDa band identified from Western blotting (data not shown). Whilst the PPG5/10 antibody did not perform well by Western blot, by RIME it detected ERβ with 25% coverage, with ERβ ranking 3^{rd} in the list of identified peptides, suggesting differences in the specificity of this antibody from one experimental method to another. Interestingly, PPG5/10 was previously validated for IHC in a doxycycline-inducible U2OS-ERβ cell line, developed using the same plasmids as the MDA-MB-231-ERβ cell line used here [Wu et al., 2012]. The Abcam 288 [14C8] antibody is a very commonly used ERβ antibody (Figure 3.1), which performed well by Western blotting and had the best antibody coverage by RIME (31.9%). However ERβ ranked 20^{th} in the list of identified peptides when using Abcam 288 [14C8], suggesting that this antibody might be pulling-down additional non-specific proteins. The CWK-F12 antibody had 17.7% coverage, with ERβ ranking 2^{nd} in the list of purified peptides. The MC10 antibody had the second-greatest coverage (28.2%) with ERβ ranking 1^{st} in the list of identified peptides.

Identification of known ERβ interactors by RIME

When the ERβ-specific peptides identified by each antibody (excluding NCL-ER-BETA) were overlapped, the only commonly identified protein was ERβ itself. The two antibodies that shared the greatest degree of overlap in terms of specific interacting peptides were MC10 and CWK-F12, with 32 common proteins identified (Figure 3.5). Two known ERβ-interacting proteins were identified in this list, heat-shock protein 90 (HSP90) [Powell et al., 2010] and ATP-dependent RNA helicase DDX54 (DDX54) [Rajendran et al., 2003], giving additional confidence in the use of RIME for antibody validation. Altogether, the degree of overlap with respect to the identified proteins between MC10 and CWK-F12 antibodies, combined with the results from Western blotting, resulted in selection of these antibodies for further experiments.



Accession	Protein
P08238	**Heat shock protein HSP 90-beta (HS90B)**
P08621	U1 small nuclear ribonucleoprotein (RU17)
P09429	High mobility group protein B1 (HMGB1)
P13639	Elongation factor 2 (EF2)
P14618	Pyruvate kinase isozymes M1/M2 (KPYM)
P15880	40S ribosomal protein S2 (RS2)
P23396	40S ribosomal protein S3 (RS3)
P46777	60S ribosomal protein L5 (RL5)
P51991	Heterogeneous nuclear ribonucleoprotein A3 (ROA3)
P62249	40S ribosomal protein S16 (RS16)
P62851	40S ribosomal protein S25 (RS25)
P62917	60S ribosomal protein L8 (RL8)
P62937	Peptidyl-prolyl cis-trans isomerase A (PPIA)
P83731	60S ribosomal protein L24 (RL24)
P84103	Serine/arginine-rich splicing factor 3 (SRSF3)
Q01130	Serine/arginine-rich splicing factor 2 (SRSF2)
Q05519	Serine/arginine-rich splicing factor 11 (SRS11)
Q08211	ATP-dependent RNA helicase A (DHX9)
Q08945	FACT complex subunit SSRP1 (SSRP1)
Q13263	Transcription intermediary factor 1-beta (TIF1B)
Q13601	KRR1 small subunit processome component homolog (KRR1)
Q14978	Nucleolar and coiled-body phosphoprotein 1 (NOLC1)
Q15397	Pumilio domain-containing protein KIAA0020 (K0020)
Q8IY81	Putative rRNA methyltransferase 3 (RRMJ3)
Q8TDD1	**ATP-dependent RNA helicase DDX54 (DDX54)**
Q8WTT2	Nucleolar complex protein 3 homolog (NOC3L)
Q92731	**Estrogen receptor beta (ESR2)**
Q9NV31	U3 small nucleolar ribonucleoprotein protein IMP3 (IMP3)
Q9NY93	Probable ATP-dependent RNA helicase DDX56 (DDX56)
Q9Y383	Putative RNA-binding protein Luc7-like 2 (LC7L2)
Q9Y3T9	Nucleolar complex protein 2 homolog (NOC2L)
Q9Y5B9	FACT complex subunit SPT16 (SP16H)

Figure 3.5: **ERβ interactors identified by RIME**. ERβ was the only common protein identified by all antibodies (excluding NCL-ER-BETA). CWK-F12 and MC10 had the greatest degree of overlap with respect to common proteins identified. Two known ERβ interactors were identified; HSP90 and DDX54. Venn diagram constructed using online tool from Bioinformatics & evolutionary genomics (www.bioinformatics.psb.ugent.be/webtools/Venn).

3.2.4 Optimisation of MC10 and CWK-F12 ERβ antibodies for Immunohistochemistry

With Dr. J. Miller, Histopathology Core Facility, CRUK Cambridge Institute.

In light of the data showing MC10 and CWK-F12 specificity for ERβ using Western blotting (Figure 3.3) and RIME (Figure 3.4), these antibodies were optimised for IHC using paraffin-embedded pellets of MDA-MB-231-ERβ+ and MDA-MB-231-ERβ- cells (Figure 3.6). Sections from the MDA-MB-231-ERβ+ and MDA-MB-231-ERβ- cell pellets were stained with MC10 diluted at 1:100 in standard Bond diluent. Three antigen retrievals were tested; Sodium Citrate for 20 minutes at 100°C, Tris EDTA for 20 minutes at 100°C and an enzyme digestion for 10 minutes at 37°C. For the latter, Leica's Bond enzyme concentrate, which contains a proteolytic enzyme (17 mg/mL) and stabiliser, diluted at 1:167 was used. No primary controls were tested for each of the retrieval conditions. As the enzyme retrieval was negative, heat induced epitope retrieval was used. Of these, Tris EDTA-treated retrieval gave better differentiation between signal versus background. The CWK-F12 antibody was tested with the same conditions as for MC10 but using a 1:250 dilution of the primary antibody based on previously published literature [Choi et al., 2001]. Good differentiation was achieved with both heat-induced epitope retrievals and no signal was detected with the enzyme pre-treatment. Once optimised, both antibodies showed strong nuclear staining in the MDA-MB-231-ERβ+ cells, with no nuclear staining in MDA-MB-231-ERβ- cells. In both ERβ+ and ERβ- conditions, the cytoplasmic staining with CWK-F12 was minimal to mild, confirming its specificity to ERβ in the IHC context. As the CWK-F12 antibody produced lower background and stromal staining than the MC10 antibody, it was preferred for further ERβ IHC studies.

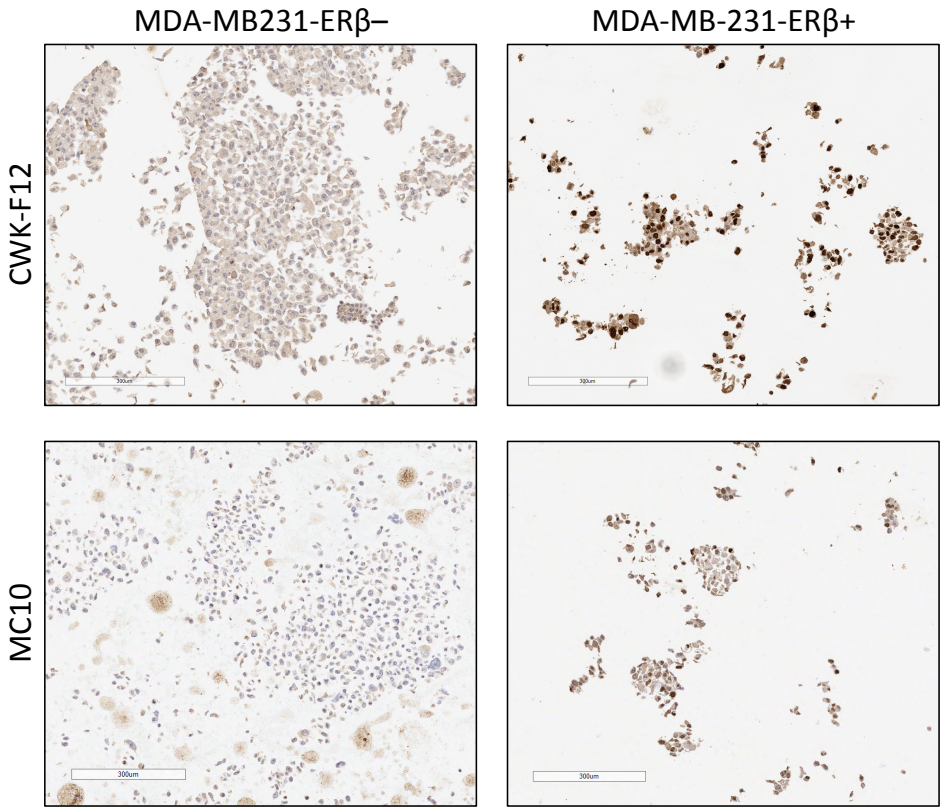


Figure 3.6: **Optimisation of CWK-F12 and MC10 ERβ antibodies in MDA-MB-231-ERβ cell pellets**. Both antibodies gave strong nuclear staining in MDA-MB-231-ERβ+ cells, which was absent from the MDA-MB-231-ERβ- control, confirming the specificity of both antibodies to ERβ. Cytoplasmic background staining was lower with the CWK-F12 antibody than the MC10, therefore it was used in further IHC analyses.

3.3 Summary

In view of the known issues with the specificity of commonly used ERβ antibodies [Choi et al., 2001; Hartman et al., 2012; Skliris et al., 2002; Weitsman et al., 2006] we designed a process for testing and validating ERβ antibodies in a manner that did not rely upon *a priori* assumptions regarding the expression of ERβ in tissues or cell line models.

The MDA-MB-231-ERβ cell line, with doxycycline-inducible ERβ expression was

validated by RT-qPCR, with dose-dependent induction of ERβ mRNA levels. Six commonly used, commercially available and two non-commercially available ERβ antibodies were tested for specificity to ERβ protein by Western blot and RIME using MDA-MB-231-ERβ- and MDA-MB-231-ERβ+ cells as negative and positive controls respectively. NCL-ER-BETA antibody was shown to yield protein bands of the appropriate size to be ERβ, but no difference was present between ERβ- and ERβ+ conditions, confirming that this antibody is not specific to ERβ. PPG5/10 antibody did not yield protein bands of appropriate size to be ERβ, demonstrating that this antibody is not useful for Western blotting of ERβ protein. GeneTex 70182 and Millipore 06-629 antibodies appeared to detect ERβ protein, with differential signal between ERβ- and ERβ+ conditions, however there were multiple other non-specific bands detected, which may confuse interpretation of data generated with these antibodies. MC10, CWK-F12, Abcam 288 [14C8] and sc8974 antibodies detected a protein band of appropriate size for ERβ, with differential signal between ERβ- and ERβ+ conditions, confirming their specificity to ERβ by Western blotting.

RIME confirmed that the NCL-ER-BETA antibody is not specific to ERβ, as no ERβ peptides were purified by this antibody. As this antibody is very commonly used in the published literature [Ellem et al., 2014; Hussain et al., 2012; McPherson et al., 2007, 2010; Oliveira et al., 2007; Umekita et al., 2006; Yang et al., 2015; Zellweger et al., 2013] it is likely to be the source of much of the controversy regarding the role and tissue expression profile of ERβ. All other antibodies, including PPG5/10 purified ERβ with varying affinity and specificity, as demonstrated by the mean number of unique ERβ peptides purified and the ERβ ranking of each antibody. Known ERβ-interacting proteins were identified in the RIME data, validating the findings. CWK-F12 and MC10 antibodies shared the greatest degree of overlap in terms of interacting proteins, and were therefore selected for use in subsequent experiments. The CWK-F12 antibody was optimised and validated for IHC use in MDA-MB-231-ERβ- and MDA-MB-231-ERβ+ cell pellets.

Using a robust and systematic methodology, this data has clearly demonstrated

wide-ranging variability of ERβ antibodies to detect ERβ protein, and has now identified reliable antibodies to be used in future applications.

Chapter 4

Establishing an experimental model for the study of Estrogen Receptor Beta

4.1 Introduction

4.1.1 Essential background information

Preclinical models are an essential component of biomedical research as they permit extensive study of the characteristics and molecular mechanisms of a disease system without the need for utilising precious primary human material. Cell lines form a substantial part of the available preclinical models currently in use [Masters, 2000]. The first cancer cell line model was developed in 1953 from an epidermoid carcinoma of the cervix and was used in the development of the polio vaccine [Scherer et al., 1953]. The first prostate cancer cell line, DU145 was developed in 1978 [Stone et al., 1978], followed by PC3 in 1979 [Kaighn et al., 1979]. Both DU145 and PC3 were developed from metastatic deposits, both are androgen receptor (AR) negative and do not respond to androgen stimulation or inhibition. The LNCaP cell line was isolated from a lymph node metastasis and became the first AR positive, androgen-sensitive cell line to be derived [Horoszewicz et al., 1980]. It has since become the 'workhorse' of prostate cancer research focusing on early stage disease, with a large number of studies published

annually using LNCaP cells as an experimental model system.

There is a substantial body of literature on the function and mechanisms of action of ERβ in prostate and breast cancer, derived from studies conducted in these and other immortalised cancer cell lines [Abd Elmageed et al., 2013; Al-Bader et al., 2011; Bouchal et al., 2011; Chen et al., 2009; Colciago et al., 2014; Dey et al., 2012, 2014; Dondi et al., 2010; Ellem et al., 2014; Fuqua et al., 1999; Hinsche et al., 2015; Holbeck et al., 2010; Kim et al., 2002a,b; Lau et al., 2000; Mak et al., 2013; Nakajima et al., 2011; Shaaban et al., 2003; Skliris et al., 2002; Veldscholte et al., 1990; Weng et al., 2013; Yang et al., 2012, 2015; Zhou et al., 2012]. There are, however discrepancies between the reported expression profiles of ERβ in individual cell lines [Holbeck et al., 2010; Nakajima et al., 2011] and furthermore, some authors have reported directly opposing roles for ERβ in studies conducted in the same prostate cancer cell line (LNCaP) [Kim et al., 2002b; Yang et al., 2012]. In the breast cancer context, the MCF-7 cell line has been reported to express endogenous ERβ [Al-Bader et al., 2011; Fuqua et al., 1999; Hinsche et al., 2015; Shaaban et al., 2003; Skliris et al., 2002]. However, it is interesting to note that mechanistic studies to determine the role of ERβ, conducted in wild-type MCF-7 cells are lacking.

Many previous studies of ERβ in prostate cancer have been conducted using the PC3 cell line [Dondi et al., 2010; Holbeck et al., 2010; Nakajima et al., 2011; Piccolella et al., 2014]. This was initially isolated from a bone metastasis of a poorly differentiated prostate adenocarcinoma [Kaighn et al., 1979] and is a commonly used model of advanced or castrate-resistant prostate cancer. With regard to nuclear steroid hormone receptors, PC3 is thought to express ERα and ERβ but not AR (determined by Western blotting and RT-qPCR) [Holbeck et al., 2010; Nakajima et al., 2011]. This has led many to regard PC3 as a model of neuroendocrine differentiated prostate cancer [Gururajan et al., 2015; Tai et al., 2011], a rare subtype of prostate cancer that rarely occurs *de novo*, but which may arise as a result of selection pressure in response to ADT in advanced disease [Beltran et al., 2012]. The lack of AR expression is a limitation of this cell line model, as even in its advanced, castrate-resistant stage, prostate

cancer tissue continues to be driven by AR and is characterised by altered AR signalling. This may be as a result of AR amplification [Bubendorf et al., 1999], mutation, stabilisation of the AR protein [Holzbeierlein et al., 2004] or generation of constitutively active splice variant isoforms [Dehm et al., 2008; Hu et al., 2012]. Consequently, PC3 bears little resemblance to either early or late stage clinical disease and is, in any case, not useful for examining the question of nuclear receptor crosstalk between ERβ and AR, which is thought to be an important phenomenon in cancer [Grubisha and DeFranco, 2013; Rizza et al., 2014; Yang et al., 2012] and will be the focus of the studies presented in Chapter 5 of this thesis.

4.1.2 Aims

Many of the previously published studies of ERβ in prostate and breast cancer have relied upon antibody-dependent techniques to determine the molecular mechanisms of ERβ in cancer cell lines. In light of our findings regarding antibody specificity (Chapter 3), it was necessary to undertake a fresh characterisation of a panel of commonly used prostate cancer cell lines using the validated ERβ antibodies, with the aim of selecting an appropriate experimental model to study ERβ. When it became clear that none of the standard models expressed ERβ, a stable, doxycycline-inducible LNCaP-ERβ cell line was developed with the aim of investigating the role of ERβ in prostate cancer development and progression.

4.1.3 Key Findings

1. A panel of prostate cell line models commonly used to study ERβ were found to lack ERβ protein expression. With the exception of PC3, none of the cell line models expressed any detectable ERβ mRNA.

2. Further assessment of the most commonly studied cell line models in the ERβ literature by RIME and PRM confirmed absence of ERβ expression.

3. The breast cancer cell line MCF-7 does not express ERβ mRNA or protein using multiple experimental approaches.

4. Expression of ERβ in prostate tissue was confirmed, demonstrating variable ERβ expression in different grades of prostate cancer tissue and thus reaffirming the rationale for investigating ERβ as a putatively important factor.

5. The stable, doxycycline-inducible LNCaP-ERβ cell line was developed and ERβ expression confirmed by multiple experimental approaches.

6. A number of proteins known to interact with ERβ and other nuclear steroid hormones were identified by RIME, providing a preliminary insight into the ERβ protein interactome in this cell line model.

4.2 Results

4.2.1 Assessment of prostate cell lines for ERβ expression

A panel of genotyped prostate cell lines commonly used to study ERβ biology, including the benign cell line PNT1a [Mak et al., 2013, 2015a,b], was assessed for expression of ERβ mRNA and protein. Protein lysate and RNA from MDA-MB-231-ERβ+ and MDA-MB-231-ERβ- cells were used as positive and negative controls respectively. Using the validated CWK-F12 ERβ antibody, none of the cell lines expressed any ERβ protein detectable by Western blotting (Figure 4.1A). With the exception of PC3, which was shown to express a low level of ERβ mRNA, none of the prostate cell lines expressed ERβ mRNA (Figure 4.1B).

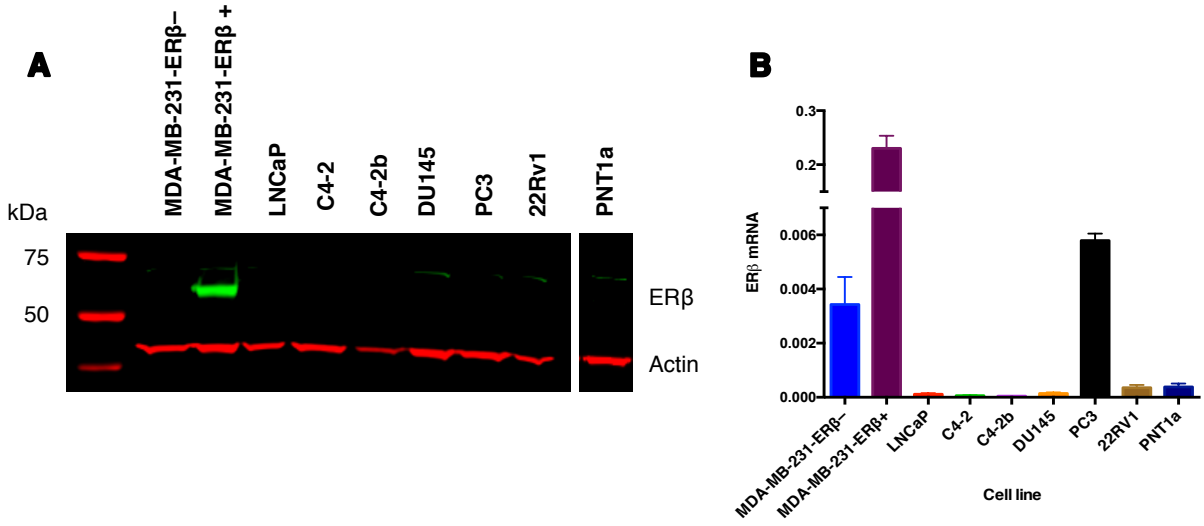


Figure 4.1: **Assessment of prostate cell lines for ERβ expression**. Prostate cell lines lack ERβ expression at (A) protein and (B) mRNA level, with the exception of PC3, which expressed a low level of ERβ mRNA (Data are mean + S.D. of technical triplicate experiments).

Given the significant number of studies previously published using the LNCaP, PC3 and PNT1a cell lines, these were further assessed for ERβ expression by RIME using the validated MC10 ERβ antibody (Figure 3.4). As E2F1 is a ubiquitously expressed protein, it was included as a positive control. No ERβ

peptides were identified in any of these cell lines across two independent biological replicate experiments (Figure 4.2).

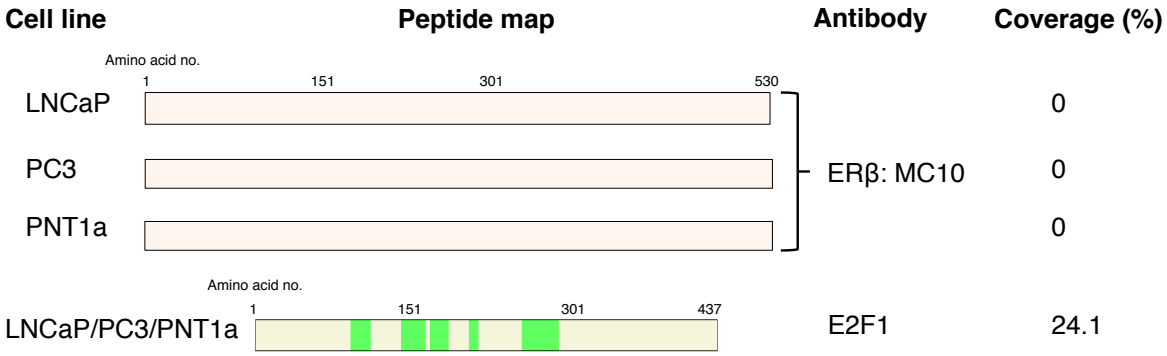


Figure 4.2: **Assessment of ERβ expression in LNCaP, PC3 and PNT1a prostate cell lines by RIME**. ERβ protein was undetectable by RIME in LNCaP, PC3 or PNT1a prostate cells.

Finally, Parallel Reaction Monitoring (PRM), an antibody-independent, targeted proteomics approach [Gallien et al., 2012] was used in LNCaP and PNT1a cell lines to search for expression of ERβ protein (Figure 4.3 and figure 4.4). Two peptides unique to ERβ were identified from the published sequence of ERβ (Peptide 1: SLEHTLPVNR; Peptide 2: SSITGSECSPAEDSK. The location of the peptides within the full-length ERβ protein is shown in figure 4.5). Heavy-labelled versions of these peptides were generated and 'spiked in' to the nuclear pellet samples collected from the cell lines. The heavy-labelled peptides were used to generate an ERβ-specific peptide fragmentation signature on MS, which then served as a positive control for identification of the light, unlabelled, endogenous protein in the sample. The technique was optimised using MDA-MB-231-ERβ+ and MDA-MB-231-ERβ- cells as additional positive and negative controls respectively.

No ERβ peptides were identified in the MDA-MB-231-ERβ- negative control, whereas a clear fragmentation pattern for each of the two endogenous (light) ERβ-unique peptides was identified in the MDA-MB-231-ERβ+ cells. This finding

further validated the results of the antibody validation experiments described in Chapter 3 by providing additional, antibody-independent verification of inducible ERβ expression in this experimental model. When using these validated ERβ PRM probes, no endogenous (light) ERβ peptides were identified in either the LNCaP or PNT1a cell lines. Taken together, these data confirm that a number of prostate cell lines that have been used extensively to study ERβ, lack detectable expression of ERβ.

4.2.2 Assessment of breast cancer cell line MCF-7 for ERβ expression

These data from the prostate cell line models were somewhat surprising, given the large number of studies of ERβ previously published using them as experimental models [Bouchal et al., 2011; Chen et al., 2009; Colciago et al., 2014; Dey et al., 2012, 2014; Ellem et al., 2014; Holbeck et al., 2010; Kim et al., 2002a,b; Lau et al., 2000; Mak et al., 2013, 2015a,b; Nakajima et al., 2011; Shaaban et al., 2003; Veldscholte et al., 1990; Weng et al., 2013; Yang et al., 2012, 2015; Zhou et al., 2012]. We then decided to assess the MCF-7 breast cancer cell line for ERβ expression using the same multimodal approach. This was done for two reasons, firstly; to test the approach in a different disease model and ensure that our data were not reflecting an 'unknown prostate-specific' effect that had not been controlled for. Secondly; ERβ is implicated as an important factor in breast cancer [Gruvberger-Saal et al., 2007; Jonsson et al., 2014; Rizza et al., 2014; Smart et al., 2013] with a number of studies demonstrating ERβ expression in MCF-7 cells [Al-Bader et al., 2011; Fuqua et al., 1999; Hinsche et al., 2015; Shaaban et al., 2003; Skliris et al., 2002]. It was thought, therefore that MCF-7 could be a useful model system to begin generating data on ERβ's mechanisms of action at a genomic level where little is currently known.

Genotyped MCF-7 cells were assessed for ERβ expression by RT-qPCR (for mRNA), as well as Western blotting, RIME and PRM (for protein) using MDA-MB-231-ERβ+/- cells as positive and negative controls (Figure 4.6). These data conclusively demonstrate that these MCF-7 cells do not express ERβ as assessed

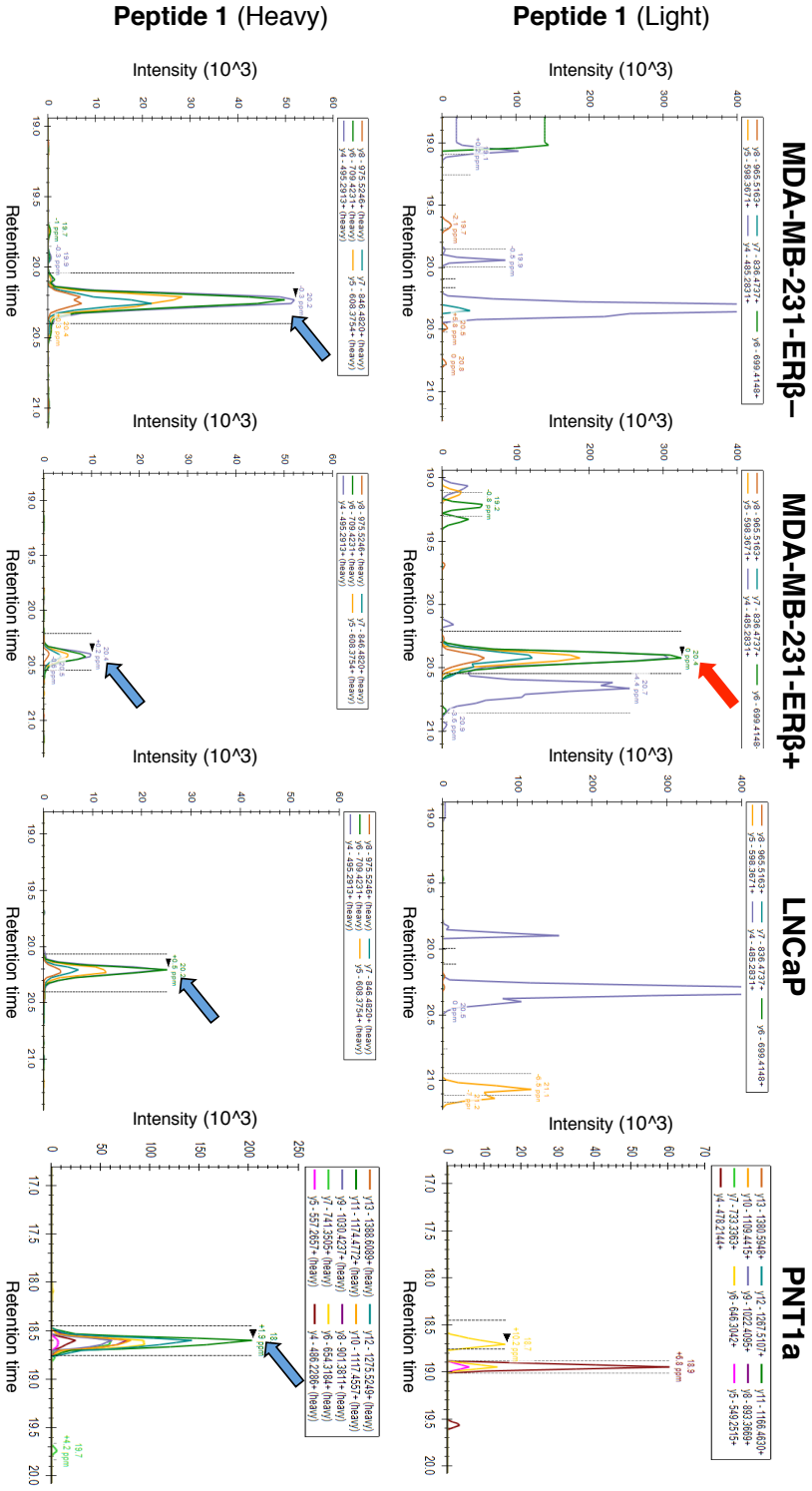


Figure 4.3: **PRM confirms that LNCaP and PNT1a do not express ERβ (Peptide 1).** The 'spiked-in', heavy labelled peptides (blue arrows) show the same retention time and fragmentation pattern as the light (endogenous) version of the peptide (red arrows), which was only identified in the MDA-MB-231-ERβ positive control and not in LNCaP or PNT1a cells.

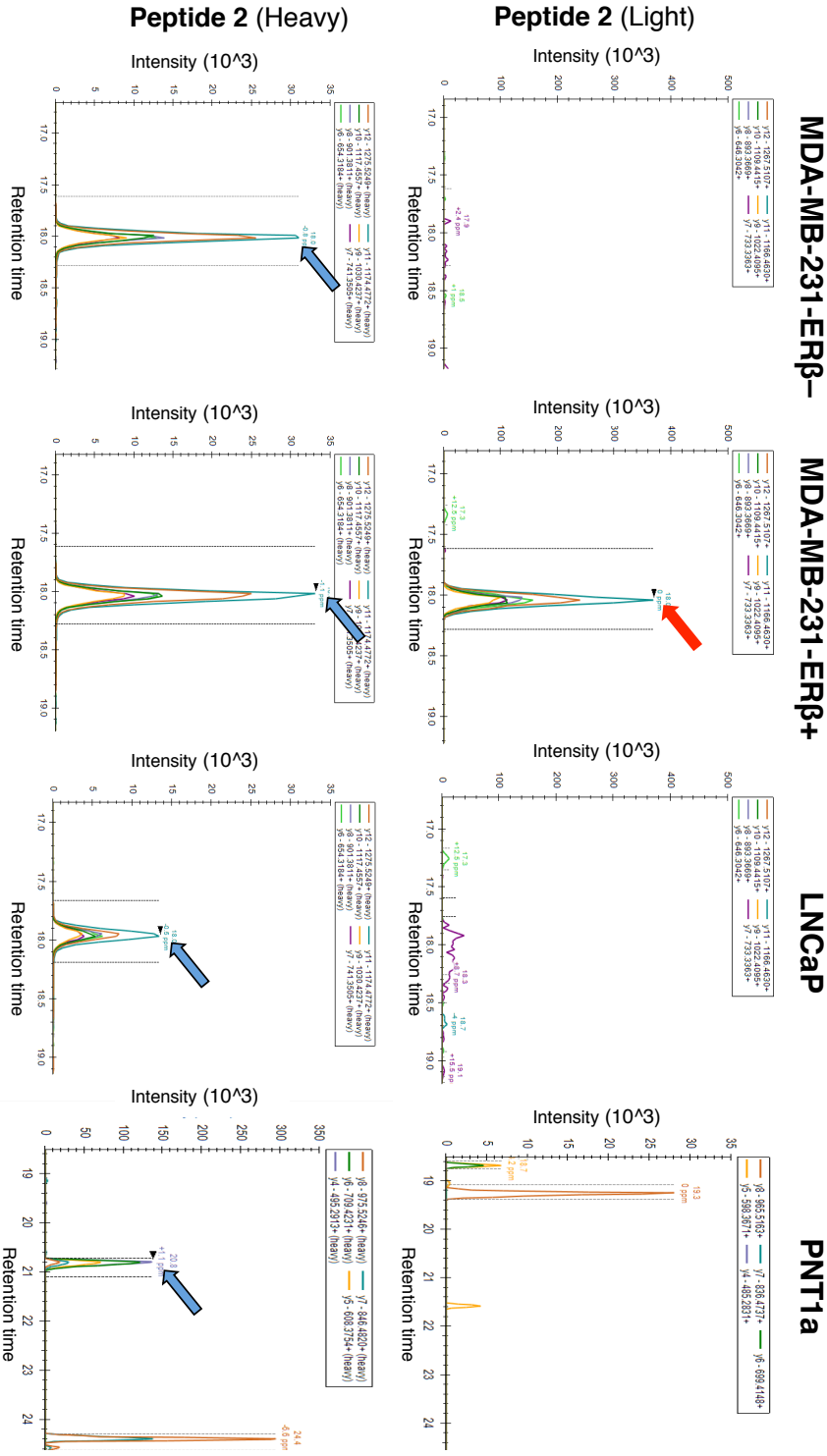


Figure 4.4: **PRM confirms that LNCaP and PNT1a do not express ERβ (Peptide 2).** The second peptide unique to ERβ (SSITGSECSPAEDSK) was also only identified in the MDA-MB-231-ERβ positive control and not in the LNCaP and PNT1a samples.

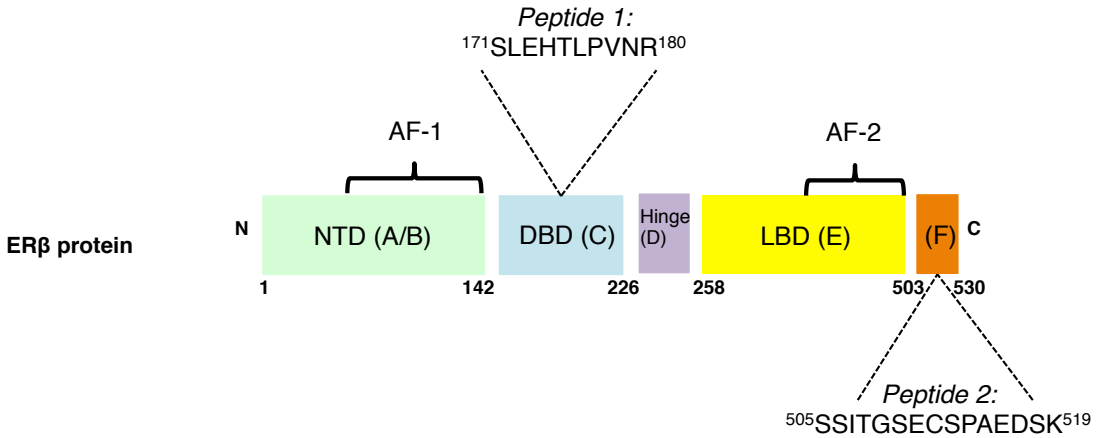


Figure 4.5: **Location of PRM peptides within full-length ERβ protein** Peptide 1 is from the DBD, common to all ERβ isoforms, whereas peptide 2 is specific to wtERβ.

by multiple experimental approaches. This cell line is therefore not an appropriate model for the study of ERβ.

It is recognised that there is some variability in immortalised cell lines across passage numbers, which may account for some of the conflicting data from one study to another generated from the same cell line [Masters, 2000]. However, having shown with multiple experimental approaches that these early passage and genotype-verified LNCaP and MCF-7 cells do not express ERβ, it is likely that much of the conflicting data regarding the role and expression of ERβ in prostate and breast cancer has arisen as a consequence of using poorly validated antibodies on these models. This is clearly demonstrated in figure 4.7, which compares Western blots of the same LNCaP and MCF-7 nuclear lysates probed with the validated CWK-F12 ERβ antibody and the commonly used but non-specific NCL-ER-BETA antibody (Figure 3.4). This non-specific ~59 kDa band detected by NCL-ER-BETA may be the source of much of the controversy surrounding the study and characterisation of ERβ in cell line models and tissue.

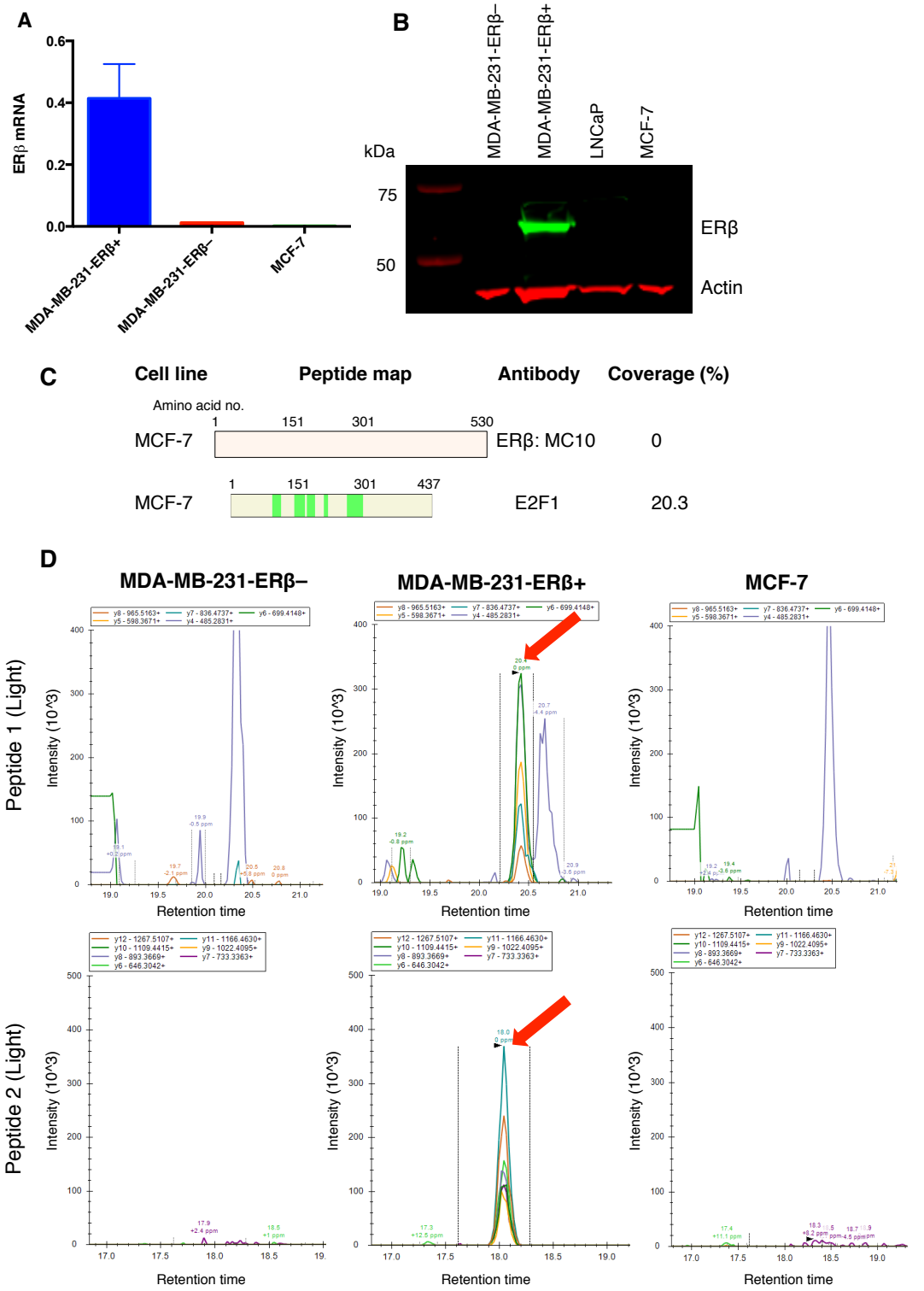


Figure 4.6: **Multimodal assessment of MCF-7 cells confirms no expression of ERβ.** Using MDA-MB-231-ERβ+/- cells as controls, MCF-7 cells were found to have no ERβ expression at (A) mRNA level (qRT-PCR) or protein level by (B) Western blotting, (C) RIME or (D) PRM. Red arrows indicate the endogenous (light) ERβ-specific peptides detected by MS in the MDA-MB-231-ERβ+ positive control.

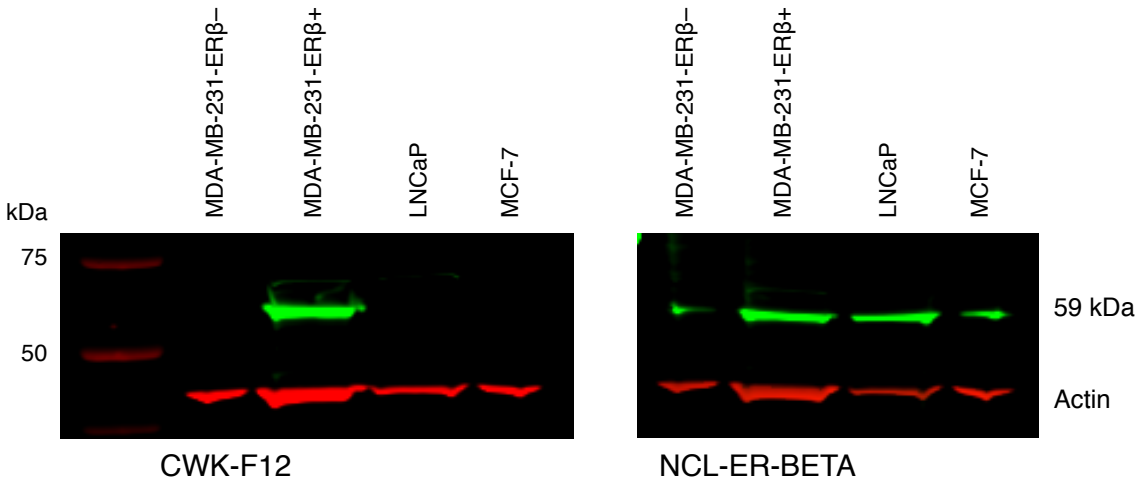


Figure 4.7: **Direct comparison of CWK-F12 and NCL-ER-BETA antibodies on LNCaP and MCF-7 nuclear lysates.** The NCL-ER-BETA antibody produces a band on Western blot of ∼59 kDA, of appropriate size for ERβ. However, the band is present in the MDA-MB-231-ERβ- control, confirming that the antibody is non-specific. The validated CWK-F12 ERβ antibody reveals the genuine result.

4.2.3 IHC confirms variable ERβ expression in differing grades of prostate cancer

Tissue staining assessed by Dr. A.Y. Warren, Consultant Histopathologist, Cambridge University Hospitals NHS Foundation Trust.

In light of the difficulties encountered with antibody specificity and the unsuccessful search for an appropriate experimental model in which to study ERβ in prostate cancer, it was necessary to confirm that prostate tissue does in fact express ERβ, and that some variability in tissue expression could be observed in different grades of prostate cancer. This would reassert the rationale for pursuing ERβ as a potentially important factor in the biology of prostate cancer development and progression.

A prostate TMA containing 10 cancers of differing grades and 5 benign controls

was stained for ERβ using the validated and optimised CWK-F12 ERβ antibody (Figure 4.8). This demonstrated high expression of ERβ in the basal epithelium of benign glands, with no expression in Gleason grade 3 cancer. Gleason grade 4 cancer showed weak nuclear staining of ERβ and in areas of Gleason grade 5 cancer, ERβ nuclear expression was of moderate intensity. This is in contrast to numerous previous reports, which have described an inverse correlation between ERβ expression and increasing Gleason grade of prostate cancer [Asgari and Morakabati, 2011; Attia and Ederveen, 2012; Dey et al., 2014; Horvath et al., 2001; Leav et al., 2001; Risbridger et al., 2007], but is consistent with other studies, which have reported an association between increased ERβ expression and higher Gleason grade [Zellweger et al., 2013] or increased expression of ERβ in bone and lymph node metastases [Bouchal et al., 2011; Zhu et al., 2004].

This analysis is limited by the small sample size of tissues stained and the lack of formal statistical correlation with clinico-pathological parameters. However, it serves as proof in principle that firstly; the CWK-F12 ERβ antibody can be used on clinical material and secondly; that variable expression of ERβ can be observed in differing grades of prostate cancer in keeping with some of the previously published data in the literature [Bouchal et al., 2011; Horvath et al., 2001; Zellweger et al., 2013; Zhu et al., 2004].

4.2.4 Development of the LNCaP-ERβ cell line

Having established that none of the evaluated prostate cell lines expressed ERβ, and having confirmed that ERβ is differentially expressed in benign versus malignant tissue, a stable prostate cancer cell line model was developed with doxycycline-inducible ERβ expression. As the potential interaction and genomic cross-talk between ERβ and AR is an area of great interest [Rizza et al., 2014; Yang et al., 2012, 2015; Zellweger et al., 2013], an androgen-sensitive cell line with endogenous AR expression was used as the parental cell line. The two-step, T-Rex^{TM}system as used to make the MDA-MB-231-ERβ cell line (plasmids donated by Dr. J. Hawse, Mayo Clinic, Minnesota, USA) was used in genotyped, low passage LNCaP cells. As the IHC data suggested that ERβ was highly expressed in benign disease, but

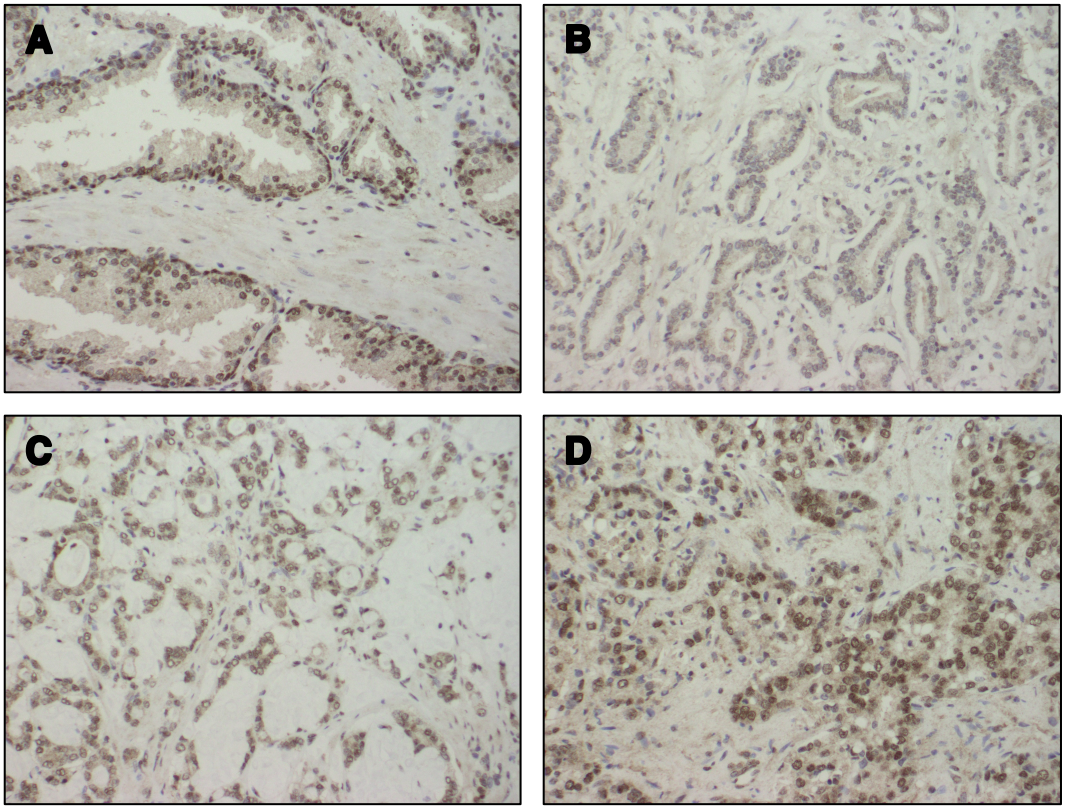


Figure 4.8: **IHC of prostate tissue with validated CWK-F12 ERβ antibody.** Variable ERβ expression was observed in differing grades of prostate cancer. ERβ was highly expressed in basal and luminal epithelial cells of benign glands (A), whereas there was no nuclear staining in Gleason grade 3 cancer (B). In Grade 4 mucinous tumor (C) and high grade tumor (D) nuclei showed weak to moderate expression of ERβ.

lost with the development of cancer, it seemed appropriate to use an androgen-sensitive cell line model representative of early stage disease. The use of a stable, inducible system allows for direct, reproducible comparisons of data obtained from the ERβ-induced conditions, eliminating the variability in the efficiency of transient transfection that may occur between replicate experiments. Furthermore, the non-induced LNCaP-ERβ- cells provide a matched negative control condition.

The procedural workflow for the development of the LNCaP-ERβ cell line model

was based on a previously published protocol [Monroe et al., 2003] and is shown in figure 4.9. LNCaP cells were transfected with the linearised pcDNA6/TR© plasmid, which contains a blasticidin resistance gene to facilitate selection of cells that have successfully incorporated the plasmid. Following 6 days of selection with 5 μg/ml blasticidin discrete cell colonies that had arisen from single cell clones were taken from the plates and seeded into 96-well plates. Approximately 80 clones of LNCaP-Tet-R cells were isolated in this way. Under continuous blasticidin selection, 20 of these clones were expanded successfully and grown up to 6-well plate format.

These 20 clones were transiently transfected with a pcDNA4/TO© plasmid containing *luciferase* to assess the activity of the Tet-R in each clone (Figure 4.10). The primary aim was to select a clone that had minimal *luciferase* expression from the 'dox-off' condition in order to create a clean system with no 'leaky' expression of ERβ. The secondary aim was to select a clone with a high -fold change in the *luciferase* signal following addition of doxycycline. The B2 clone satisfied both of these criteria, and was therefore used for subsequent transfection of the ERβ plasmid.

Several initial attempts at transfecting the pcDNA4/TO©-ERβ plasmid into the LNCaP-Tet-R B2 clone were unsuccessful. A number of different strategies were used to overcome this. Side-by-side transfections using linearised versus non-linearised plasmid, and Lipofectamine® 2000 versus Lipofectamine® 3000 were performed. Successful transfection was eventually achieved using non-linearised plasmid transfected with Lipofectamine® 2000. Further difficulties were encountered with zeocin selection of cells; treating the cells with 500 μg/ml dose as previously described [Wu et al., 2012] did not produce enough cell death to prevent plates from becoming fully confluent before selection had taken place. However, if cells were then passaged and treated with 500 μg/ml zeocin they all died.

In order to refine the zeocin selection process, a dose-response experiment was performed in the LNCaP-Tet-R B2 cell line (Figure 4.11). This demonstrated

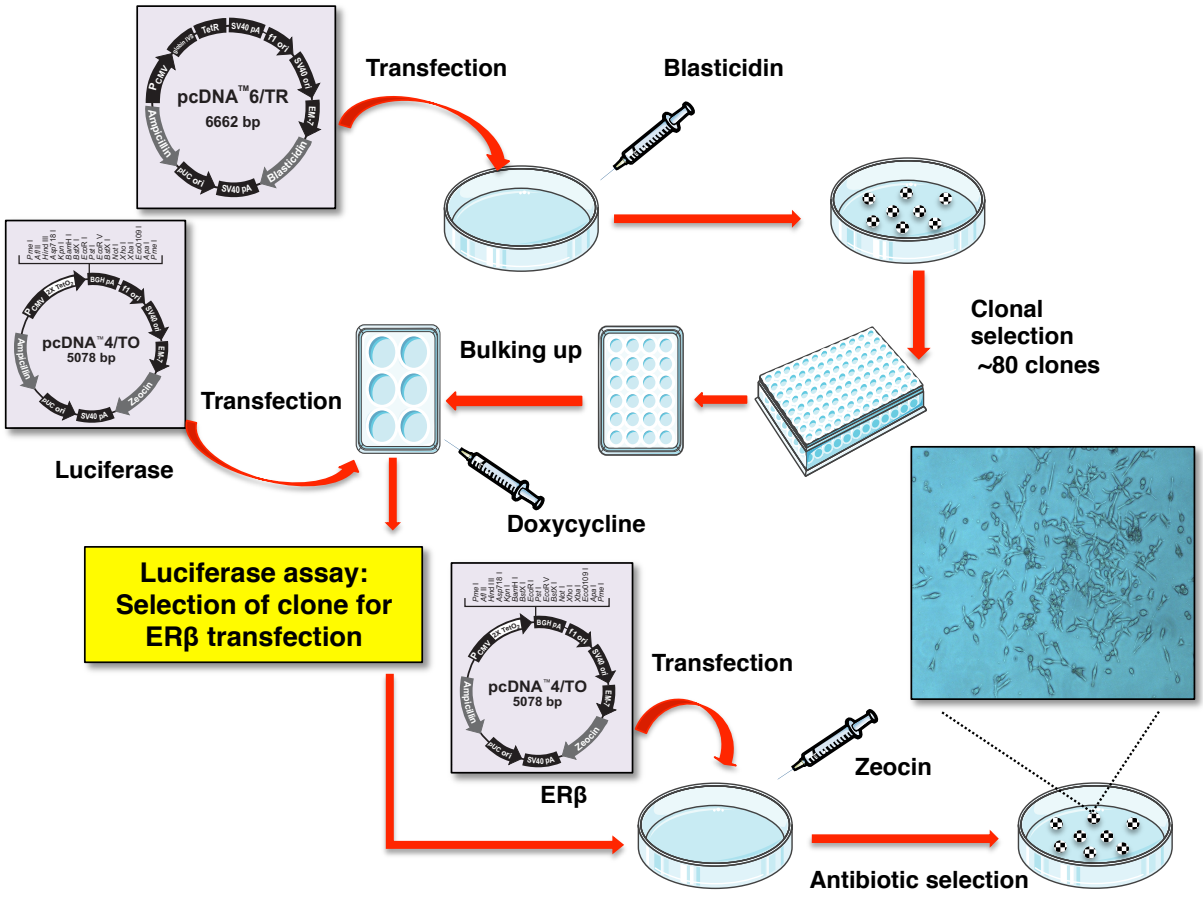


Figure 4.9: **Workflow for the development of the LNCaP-ERβ cell line.** LNCaP cells were transfected with a Tet-R construct, selected using blasticidin and replated to grow single cell colonies. Individual colonies were reseeded in 96-well plates. Expanded clones were transiently transfected with *luciferase* to measure Tet-R activity. The clone with the greatest -fold induction and lowest 'dox-off' activity was selected for transfection with ERβ-containing plasmid and cells selected with zeocin.

that 1000 μg/ml zeocin was required to select successfully transfected cells within 6 days of treatment. Lower doses did not produce any cell death, whereas the 1,250 μg/ml dose killed almost all cells. Following selection, the dose of zeocin was reduced to 250 μg/ml for maintenance of the cell line, as it was found that continuous treatment with zeocin 500 μg/ml as used in the MDA-MB-231-ERβ cell line [Wu et al., 2012] resulted in intractable cell senescence (Figure 4.12B)

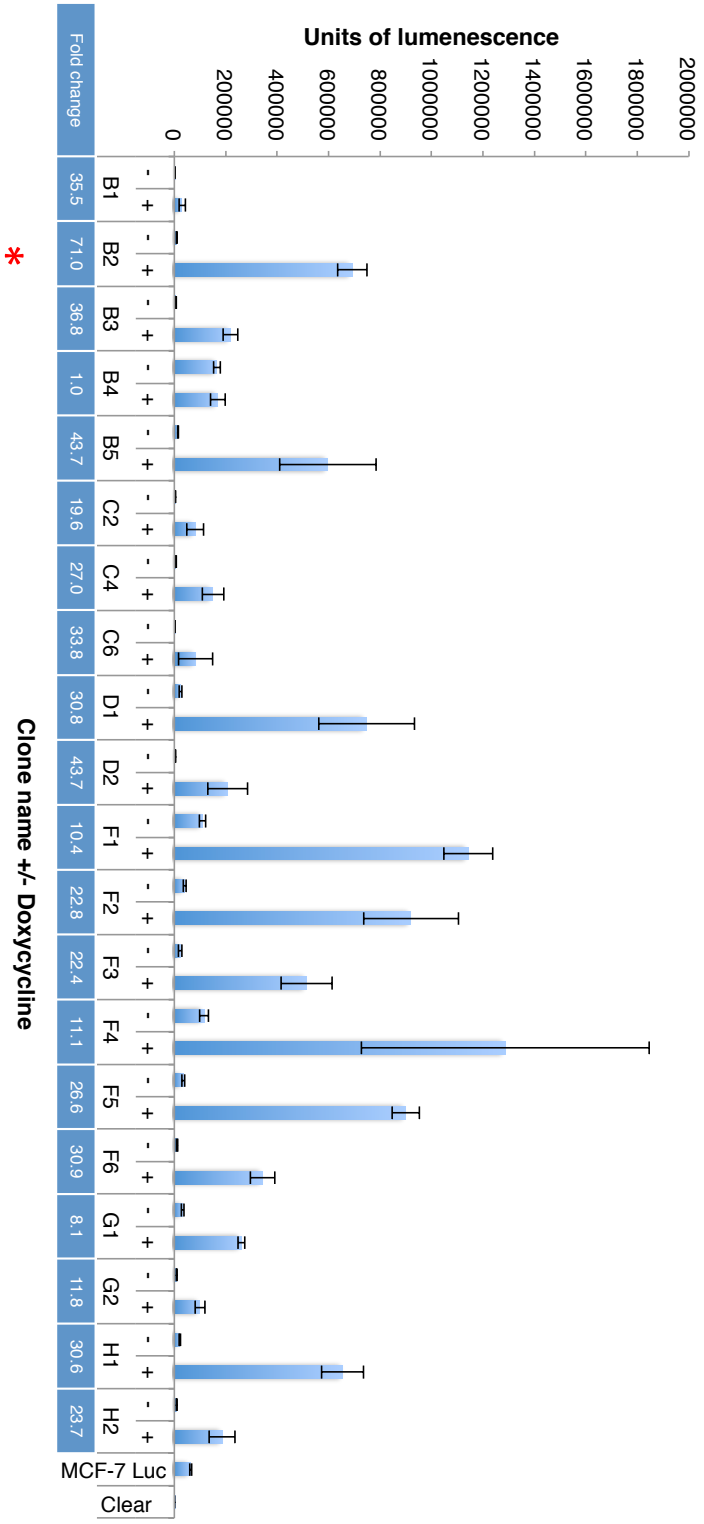


Figure 4.10: ***Luciferase* assay of LNCaP-Tet-R clones.** A total of 20 Tet-R clones were screened. The B2 clone (*) had the lowest 'dox-off' signal and the greatest -fold induction with 0.1 μg/ml doxycycline treatment. Data are mean +/- S.D. of triplicate experiments.

[Brandhagen et al., 2013]. Under the maintenance doses of blasticidin 2.5 μg/ml and zeocin 250 μg/ml cells proliferated at the same rate and were subcultured at the same ratios as parental LNCaP cells. Once proliferating, the LNCaP-ERβ cell line was morphologically identical to the parental LNCaP cell line (Figure 4.12C).

4.2.5 Characterisation of the LNCaP-ERβ cell line

The same multimodal characterisation, utilising RT-qPCR, Western blotting, RIME and PRM was then applied to the LNCaP-ERβ cell line to confirm firstly; absence of ERβ expression in the 'dox-off' condition and secondly; detectable induction of ERβ mRNA and protein following 24 hour treatment with 0.1 μg/ml doxycycline. Induction of ERβ mRNA in the LNCaP-ERβ cell line was compared to that of the MDA-MB-231-ERβ cell line (Figure 4.13A). Absolute levels of ERβ mRNA were greater in the LNCaP-ERβ+ cell line than the MDA-MB-231-ERβ+ cell line. However, due to a greater level of baseline ERβ expression in the LNCaP-ERβ- cells, the fold-change of ERβ expression with doxycycline treatment was greater in the MDA-MB-231-ERβ line than the LNCaP-ERβ cells (81 fold increase in expression versus 15 fold respectively). Induction of ERβ expression in the LNCaP-ERβ cell line had no effect on mRNA expression of AR (Figure 4.13B). Western blotting with the validated CWK-F12 ERβ antibody confirmed strong ERβ protein expression in the LNCaP-ERβ+ condition, with no detectable ERβ protein in the ERβ- condition. This result validated the induction of ERβ in the LNCaP-ERβ cell line, confirmed the specificity of this antibody, and provided additional evidence that non-induced LNCaP-ERβ cells are ERβ negative. Protein expression of AR and FOXA1, an important nuclear receptor co-factor [Carroll et al., 2005; Robinson et al., 2011, 2014], were unaltered by induction of ERβ expression (Figure 4.13C).

Induction of ERβ protein expression in the LNCaP-ERβ cell line was confirmed by PRM and RIME (Figure 4.14). In the PRM analysis, comparison of the fragmentation pattern and retention time of the heavy-labelled ERβ with that observed from the endogenous ERβ in the LNCaP-ERβ+ cells confirmed the

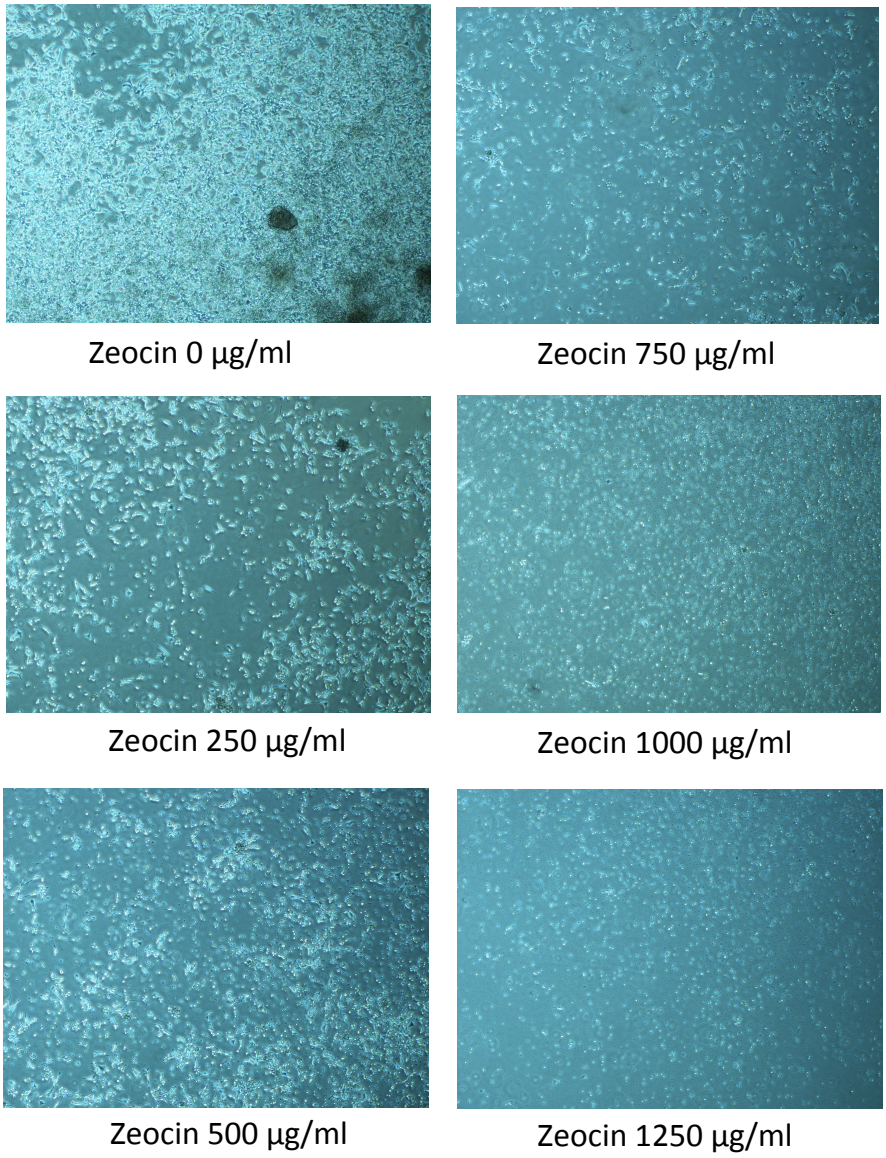


Figure 4.11: **Zeocin dose-response in LNCaP-Tet-R B2 cells.** Cells were treated with increasing doses of zeocin for 6 days. 1000 μg/ml resulted in significant cell death without killing all cells.

presence of ERβ peptide in the sample, although in lower abundance as had been detected in the MDA-MB-231-ERβ+ cells (Figure 4.3). Furthermore, only peptide 1 (SLEHTLPVNR) was detected in the LNCaP-ERβ+ cells. This is likely due to lower abundance of ERβ protein in the LNCaP-ERβ+ cell line as

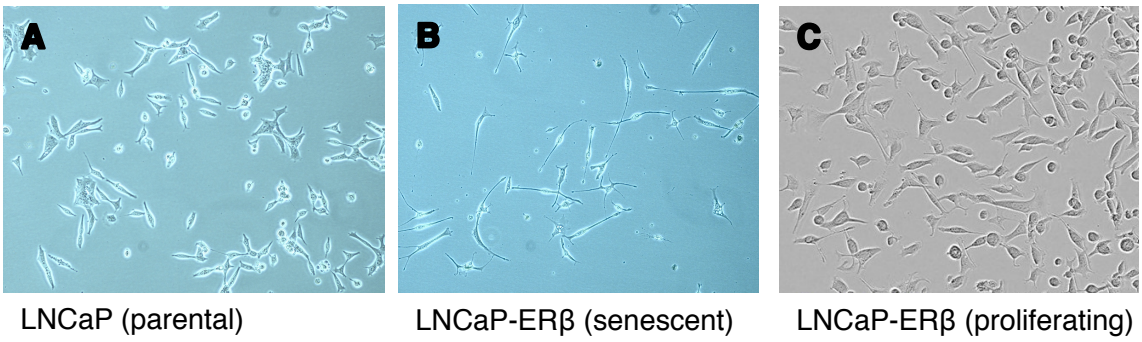


Figure 4.12: **Morphology of LNCaP-ERβ cells.** LNCaP-ERβ cells treated continuously with zeocin 500 μg/ml stopped proliferating, and developed long neurite-like projections characteristic of a senescent phenotype (B). When doses of blasticidin and zeocin were reduced (C) the LNCaP-ERβ cells behaved like parental LNCaP cells (A).

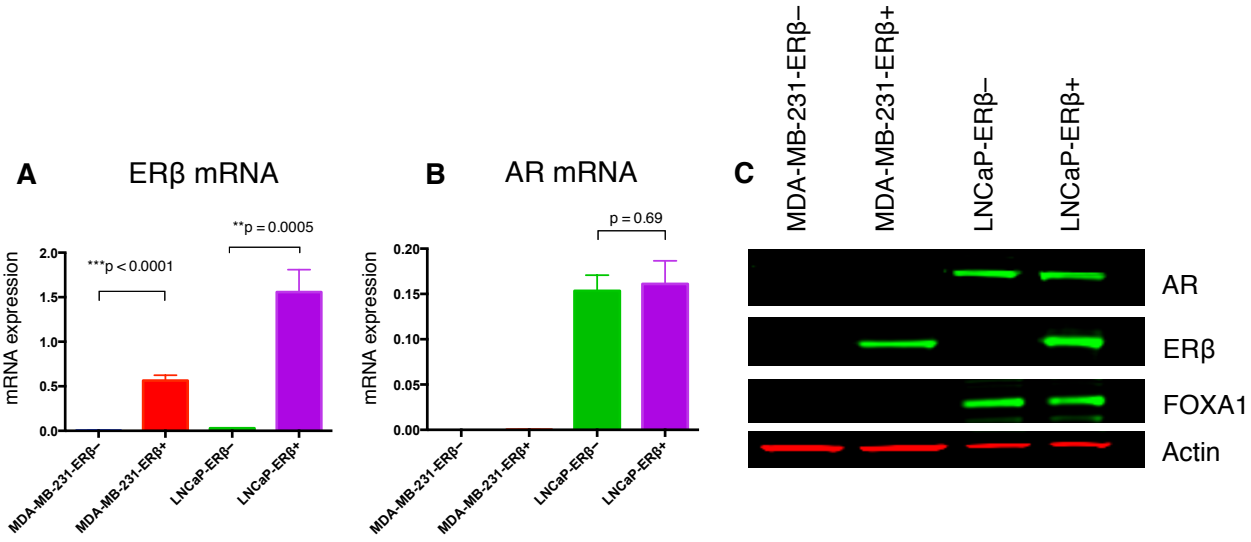


Figure 4.13: **Characterisation of LNCaP-ERβ cell line.** Doxycycline-induced expression of ERβ was confirmed in the LNCaP-ERβ cell line at (A) mRNA and (C) protein level. Comparison with the MDA-MB-231-ERβ cell line showed 15-fold ($p < 0.0001$) versus 81-fold ($p = 0.005$) increase in expression respectively. AR mRNA (B) and protein, and FOXA1 protein (C) expression were unaffected by ERβ induction. RT-qPCR data are mean + S.D. of triplicate experiments. No ERβ protein was detected in the LNCaP-ERβ- condition.

compared with the MDA-MB-231-ERβ+ cell line.

RIME and PRM analyses of LNCaP-ERβ- cells demonstrated no detectable expression of ERβ proteins in keeping with the Western blot results, confirming the robust nature of the inducible LNCaP-ERβ cell line model system. In LNCaP-ERβ+ cells, using the validated MC10 antibody, 30% peptide coverage was achieved with a mean of 14.5 peptides unique to ERβ identified.

4.2.6 The ERβ protein interactome

The ERβ protein interactome is not well characterised in any disease or physiological context. The RIME data obtained from the LNCaP-ERβ+ cells provide the opportunity for a preliminary examination of protein interactions and networks relating to ERβ in a prostate cancer context. The BioGRID database [Chatr-Aryamontri et al., 2015] was used to identify previously published interactors of proteins pulled down by ERβ RIME (Table 4.1). These included 4 known ERβ interactors; TRIM24, NRIP1, HMGB1 and HSP90, and 9 proteins known to interact with other nuclear steroid hormone receptors (discussed in section 6.3.3). Network and pathway analysis of all specific peptides purified by ERβ RIME was conducted using the STRING database [Szklarczyk et al., 2015] (Figure 4.15). A significant proportion of the proteins purified in the ERβ RIME clustered into two nodal networks. Gene ontology (GO) term analysis revealed that one node was strongly associated with RNA splicing (FDR 1.55 x 10^{-13}) and the second comprised proteins involved in translation initiation machinery (FDR 2.76 x 10^{-12}). Three other functional networks were apparent in the interacting proteins, DNA damage repair proteins (FDR 0.0145), regulation of gene expression (FDR 0.0114) and regulation of apoptosis (FDR 0.018). The four known ERβ-interacting proteins identified in the BioGRID database (TRIM24, NRIP1, HMGB1 and HSP90) were positively identified in this analysis. Interestingly, our RIME data presents evidence of direct interaction between ERβ and AR (Table 4.1), which was confirmed by the STRING analysis.

Although RIME data are not quantitative, and further validation is required to

prove physical interaction between proteins purified by RIME, these data provide an interesting insight into putative ERβ-interacting protein networks.

4.3 Summary

A panel of prostate cell lines commonly used to study ERβ were found to lack detectable ERβ protein expression by Western blotting using the validated CWK-F12 antibody. Only the PC3 cell line expressed any detectable ERβ mRNA. Further assessment of the most commonly utilised prostate cancer cell lines in the ERβ literature by RIME (LNCaP, PC3 and PNT1a) and PRM (LNCaP and PNT1a) confirmed that no ERβ protein could be detected in these cell lines using either validated antibody-based, or antibody-independent approaches. Furthermore, the breast cancer cell line MCF-7, which has also been extensively used to study ERβ was also found to lack ERβ expression using the same multimodal approach. These findings call into question any previously published data on the role of ERβ generated using these cell line models.

Differential expression of ERβ protein in benign versus malignant prostate tissue was confirmed by IHC using the validated CWK-F12 antibody, reaffirming the case for studying the role of ERβ as a putative tumour-suppressor in prostate cancer. In view of the IHC findings and the lack of an available cell line model suitable for *in vitro* mechanistic studies of the role of ERβ, a prostate cancer cell line with stable, inducible ERβ expression was developed and validated by four independent methods. Importantly, no ERβ expression was detectable in the uninduced, ERβ- condition by any technique, demonstrating that this is a robust model that will enable controlled studies of the interplay between ERβ and AR in an androgen-sensitive prostate cancer context.

A number of proteins known to interact with ERβ, along with other proteins known to interact with other nuclear steroid hormone receptors were identified by RIME in LNCaP-ERβ+ cells, further validating the model and revealing a putative network of ERβ-interacting proteins, with multiple nodes of protein networks revealed centring on RNA splicing mechanisms, translational machinery,

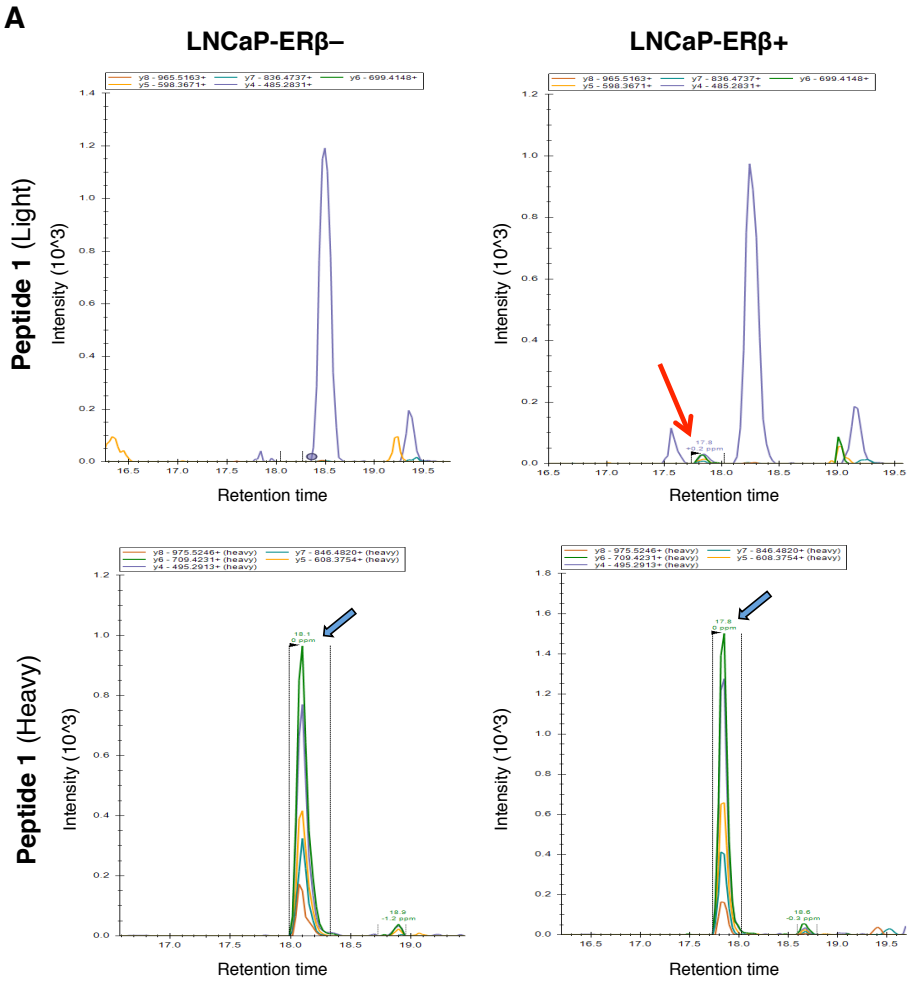
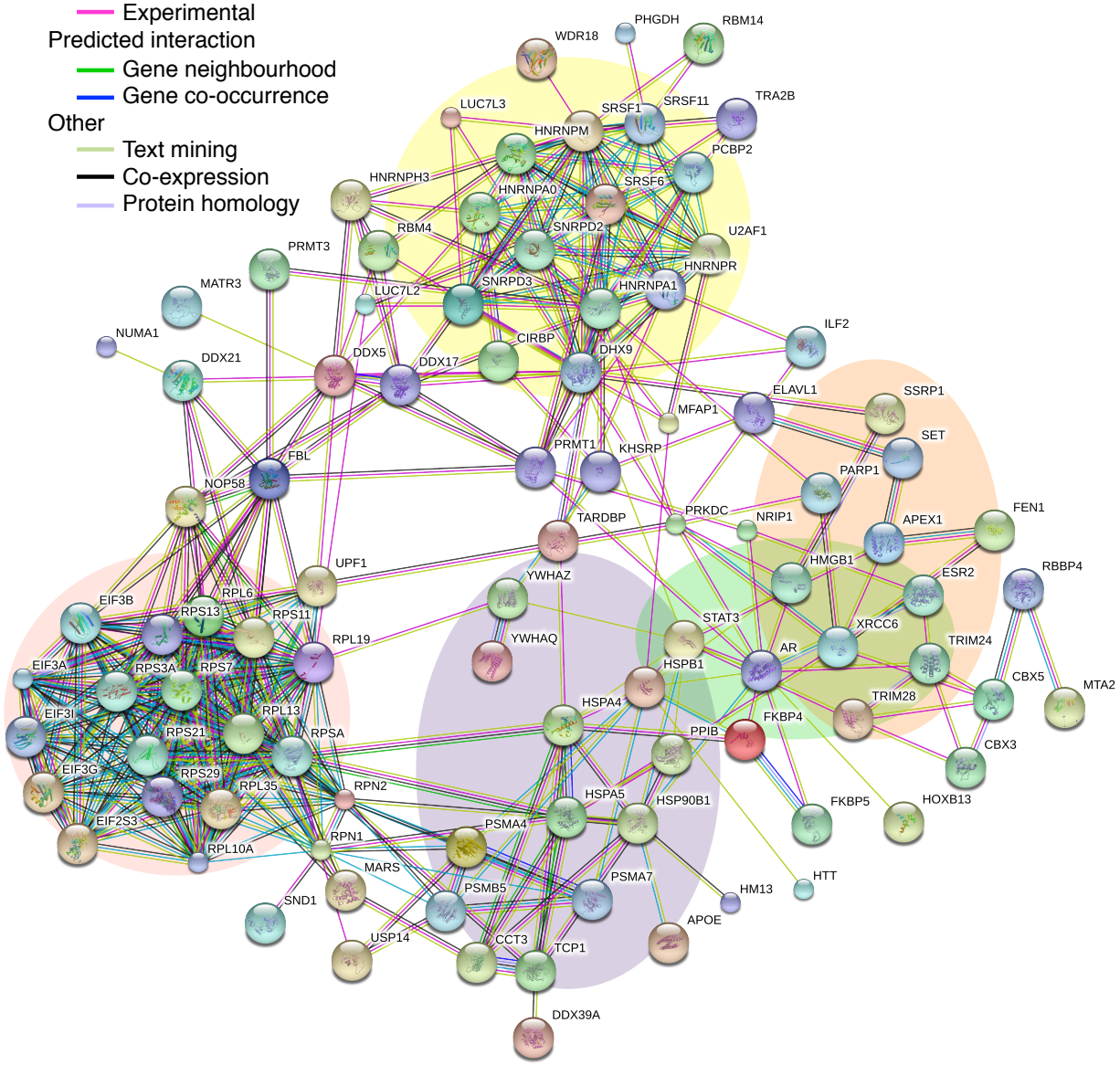


Figure 4.14: **Characterisation of LNCaP-ERβ cell line by PRM and RIME.** (A) PRM did not detect ERβ peptides in LNCaP-ERβ- cells. ERβ peptides were identified in LNCaP-ERβ+ cells (red arrow), with the same fragmentation pattern and retention time as the heavy-labelled peptide (blue arrows). (B) RIME confirmed no ERβ expression in LNCaP-ERβ- cells, but strong pull-down of ERβ in LNCaP-ERβ+ cells, with mean coverage of 30% and 14.5 unique peptides. AR acted as a positive control, with no difference in coverage or unique peptides observed between ERβ- and ERβ+ conditions.

Description	Mean coverage	Mean peptides
E3 UFM1-protein ligase 1 (**UFL1**)	33.8	22
Estrogen receptor beta (**ESR2**)	30.6	14.5
Protein arginine N-methyltransferase 3 (**ANM3**)	21.8	11
T-complex protein 1 subunit alpha (**TCPA**)	22.1	10.5
Transcription intermediary factor 1-beta (**TIF1B**)	14.7	9
Probable ATP-dependent RNA helicase (**DDX17**)	16.5	8
Signal transducer and activator of transcription 3 (**STAT3**)	14.4	8
Heterogeneous nuclear ribonucleoprotein M (**HNRNPM**)	13.4	8
Heterogeneous nuclear ribonucleoprotein A1 (**ROA1**)	25.1	7.5
Poly [ADP-ribose] polymerase 1 (**PARP1**)	8.3	7.5
Protein arginine N-methyltransferase 1 (**ANM1**)	21.7	6
Protein O-linked-mannose beta-1,4-N-acetylglucosaminyltransferase 2 (**PMGT2**)	15.6	6
Far upstream element-binding protein 2 (**FUBP2**)	12.1	6
Endoplasmin (**HSP90/ENPL**)	8.9	5.5
Staphylococcal nuclease domain-containing protein 1 (**SND1**)	6.8	5.5
Nuclear receptor-interacting protein 1 (**NRIP1/RIP140**)	6.7	5.5
Eukaryotic translation initiation factor 3 subunit A (**EIF3A**)	5.3	5.5
Creatine kinase B-type (**KCRB**)	18.6	5
Peptidyl-prolyl cis-trans isomerase (**FKBP5**)	15.5	5
WD repeat-containing protein 18 (**WDR18**)	12.6	5
Peptidyl-prolyl cis-trans isomerase (**FKBP4**)	13.3	4.5
X-ray repair cross-complementing protein 6 (**XRCC6**)	10.0	4.5
Minor histocompatibility antigen H13 (**HM13**)	12.7	4
Heat shock 70 kDa protein 4 (**HSP74**)	5.8	4
Eukaryotic translation initiation factor 3 subunit B (**EIF3B**)	4.2	3.5
DNA-dependent protein kinase catalytic subunit (**PRKDC**)	1.3	3.5
High mobility group protein B1 (**HMGB1**)	18.6	3
Transcription intermediary factor 1-alpha (**TRIM24/TIF1A**)	4.1	2
FACT complex subunit (**SSRP1**)	2.4	1.5
Homeobox protein Hox-B13 (**HOXB13**)	4.2	1
Metastasis-associated protein (**MTA2**)	2.4	1
Androgen receptor (**AR**)	2.2	1

Table 4.1: **Interacting proteins identified by ERβ RIME in LNCaP-ERβ+ cells.** All proteins identified with $\geq$5 unique peptides are shown, along with other interesting or previously published steroid hormone receptor-interacting proteins (Mean of 2 independent biological replicates).



Colour key	GO term	Count in gene set	FDR
	RNA splicing	19	1.55×10^{-13}
	Translation initiation	18	2.76×10^{-12}
	DNA repair	10	0.0145
	Positive regulation of gene expression	22	0.0114
	Regulation of apoptosis	19	0.018

Figure 4.15: **ERβ-interacting protein networks identified by RIME in LNCaP-ERβ+ cells.** Five nodes of interacting proteins are apparent, with gene ontology (GO) pathway analysis revealing functional correlations of the proteins within the nodes. (FDR, false discovery rate).

DNA damage response and apoptotic mechanisms.

Chapter 5

Genomic crosstalk between ER beta and AR

5.1 Introduction

5.1.1 Essential background information

Silencing of ERβ contributes to early events in prostate carcinogenesis

The early molecular events that drive the initial development of prostate cancer are incompletely understood [Zhou et al., 2015]. Both the *in utero* development and subsequent homeostatic regulation of the adult prostate are dependent upon DHT-induced activity of AR [Hayward et al., 1998; Pointis et al., 1979]. In benign prostate tissue, AR maintains luminal epithelial cell differentiation, possibly through a paracrine signalling network involving stromal cells [Wikstrom et al., 1999]. Therefore, in order for AR to become an oncogene and the principle driver of prostate cancer, its normal homeostatic role is disturbed. A number of processes are thought to be involved in this change; reprogramming of the AR cistrome by co-factors [Pomerantz et al., 2015], formation of fusion genes by aberrant DNA-damage response mechanisms [Mani et al., 2009] with the resulting deletion of *PTEN* leading to upregulation of the Akt/PI3K signalling pathway [Carver et al., 2009], over-expression of AR [Zhou et al., 2013] or post-translational modifications to AR [Coffey and Robson, 2012].

It has been proposed that a decrease in systemic androgen levels may be the initiating factor in this carcinogenic process [Zhou et al., 2015]. There is evidence for this at an epidemiological level as it is well-established that the peak age of prostate cancer incidence coincides with the age at which serum testosterone levels decline [Vermeulen et al., 2002]. As discussed in section 1.3, this change results in a decreased androgen:estrogen ratio, which is hypothesised to contribute to prostate cancer risk [Bosland, 2013; Ellem and Risbridger, 2007]. Decreased availability of androgen ligands leads to upregulation of AR expression in an attempt to maintain normal AR signalling. This change in AR function leads to DNA damage, through AR-mediated recruitment of topoisomerase 2β (TOP2β) resulting in double-stranded DNA breaks [Mills, 2014]. The ensuing genome instability leads to formation of E26 transformation-specific (ETS) family fusion genes such as TMPRSS2:ERG, a common genetic lesion in early prostate cancer development [Weischenfeldt et al., 2013] that results in cell cycle dysregulation, increased DNA synthesis and upregulation of proinflammatory mediators such as STAT3 and NF-κB [Mills, 2014] (Figure 5.1). As discussed in section 1.5.1, ERα and ERβ (in particular loss of ERβ expression) play critical roles with respect to inflammatory processes in the prostate that contribute to cancer development and progression (Figure 1.6), and so one could hypothesise that loss of ERβ expression in malignant transformation of the prostate may contribute to these proinflammatory changes resulting from fusion gene formation.

A second key event in the initiation of prostate carcinogenesis is loss of the tumour suppressor gene *PTEN*. This leads to upregulation of the Akt/PI3K signalling pathway, which is known to be an important driver of changes that eventually lead to CRPC [Phin et al., 2013]. As discussed in section 1.5.2, *PTEN* loss results in decreased wtERβ expression (mediated through silencing of the 0N *ESR2* promotor) and leads to predominance of the ERβ2 isoform (due to persistent activity from the 0K *ESR2* promotor) with subsequent upregulation of VEGF signalling and inhibition of caspase-9 mediated apoptosis (Figure 1.7), all of which contribute to the development and progression of prostate cancer [Dey et al., 2014; Lee et al., 2013; Leung et al., 2006b; Mak et al., 2015a; Suzuki et al.,

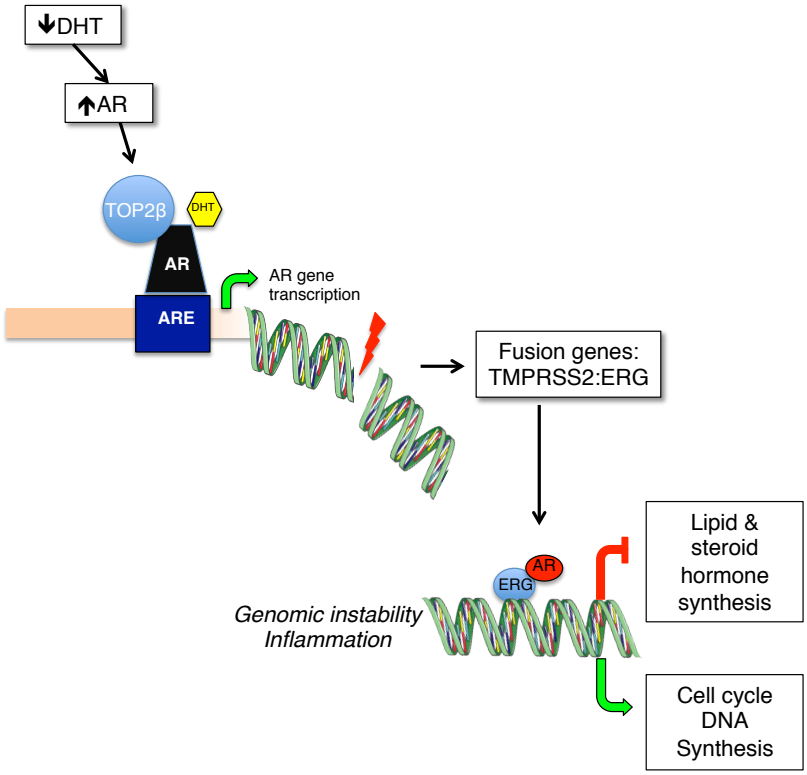


Figure 5.1: **TMPRSS2:ERG fusion gene formation drives early prostate carcinogenesis through genome instability and proinflammatory mediators.** Decreased serum androgen results in upregulation of AR expression, leading to double stranded DNA-breaks and formation of fusion genes. This drives pro-inflammatory pathways known to be related to ERβ function. Figure based on Mills 2014 and Zhou et al., 2015.

2008]. Furthermore, in a recent study that appears to mechanistically link these two early genetic lesions it has been shown that activation of ERβ2 triggers upregulation of another ETS-family fusion oncogene, TMPRSS2:ETV5a/5b in androgen deplete conditions, which in turn mediates growth and cell migration in CRPC [Kim et al., 2016].

Considering these three factors together: i) the change in the androgen:estrogen ratio; ii) the resulting change in AR activity that drives the formation of the TMPRSS2:ERG fusion gene leading to genome instability and inflammation (Figure 1.6) and iii) the loss of *PTEN* expression, which silences wtERβ expres-

sion and increases ERβ2 expression with subsequent upregulation of additional TMPRSS2:ETS-family fusion genes, we can observe that ERβ is intimately involved in each of these processes, and hypothesise that the loss of ERβ's tumour suppressive effect is a an important early event in prostate carcinogenesis. IHC data showing that ERβ is highly expressed in benign prostate epithelium, but not in cancer [Asgari and Morakabati, 2011; Bonkhoff et al., 1999; Dey et al., 2014; Horvath et al., 2001; Leav et al., 2001] would support this hypothesis.

ERβ and AR modulate each other's activity through nuclear receptor crosstalk

As discussed in section 1.5.2, there is increasing evidence to show that individual nuclear receptors do not function in isolation, but instead are interdependent and able to modulate each other's responses to stimuli [Arora et al., 2013; Mohammed et al., 2015]. Previous work in breast and prostate cancer has shown evidence of crosstalk between ERβ and AR by several mechanisms, but the exact nature of the relationship between the two receptors remains unclear [Grubisha and DeFranco, 2013; Mizokami et al., 2004; Rizza et al., 2014; Teng et al., 2014; Thelen et al., 2007; Weng et al., 2013], specifically whether ERβ regulates AR or *vice versa*. The current lack of a coherent mechanism explaining the relationship between these receptors may simply reflect the differing cell- and tissue-specific contexts in which studies have been conducted. Alternatively, confusion in the literature may have arisen as a consequence of the use of inadequately validated reagents and experimental models as demonstrated in Chapters 3 and 4 of this thesis.

In the context of prostate cancer the details of ERβ's mechanism of action must be fully understood before it can be considered as a valid therapeutic target, especially given the current disconnection between *in vitro* and *in vivo* data on the use of SERMs in prostate cancer [Kim et al., 2002a,b; Nakamura et al., 2013; Piccolella et al., 2014; Rossi et al., 2011] (Sections 1.3.3 and 1.3.4). From a clinical perspective, the key question is whether maintaining ERβ signalling can alter or reprogram the actions of AR to slow or even prevent the AR-mediated processes that drive early prostate carcinogenesis. This strategy, by modifying AR activity

early in the disease process in an *indirect* manner, may present an alternative to conventional ADT, which by directly targeting AR once its oncogenic role is already firmly established, inevitably exerts selection pressure to drive cancer evolution and the development of lethal CRPC (Section 1.1.7) [Claessens et al., 2014; Scher et al., 2004].

5.1.2 Aims

The aim of the work presented in this chapter is to understand the effect and determine the mechanisms by which ERβ influences AR-driven prostate carcinogenesis. To address this, a number of previously published clinical datasets were interrogated to look for i) patterns of ERβ expression and associations with clinical prognosis and ii) the effect of inhibiting AR with ADT on ERβ expression, with a view to determining the relationship between ERβ and AR. Secondly, in light of the previously described observations from published IHC studies showing that ERβ is highly expressed in benign prostate epithelium and lost in malignant epithelium [Asgari and Morakabati, 2011; Attia and Ederveen, 2012; Bonkhoff et al., 1999; Horvath et al., 2001], we hypothesised that loss of ERβ results in alteration of AR activity; specifically, the transition of AR from an agent of physiological homeostasis to an oncogene. We therefore decided to reintroduce ERβ into an androgen-sensitive, AR-driven prostate cancer context to determine the effect of maintained ERβ signalling on oncogenic AR. In so doing, we aimed to determine the mechanisms of crosstalk between these nuclear receptors in an early disease context. The inducible LNCaP-ERβ cell line model described in Chapter 4 was therefore used to study the effects of activated ERβ at phenotypic, genomic and transcriptional levels.

5.1.3 Key Findings

1. Interrogation of a large clinical dataset integrating gene expression data with copy number aberrations [Ross-Adams et al., 2015] shows that increased ERβ expression is associated with improved BCR-free survival and greater genome stability.

2. Interrogation of published gene expression data, combined with ERβ IHC in prostatectomy specimens from clinical trials of ADT shows that ERβ expression is inhibited by AR *in vivo*. AR knockdown in a prostate cancer cell line results in upregulation of ERβ expression confirming this finding in an *in vitro* setting.

3. Prostate cancer cell proliferation is inhibited by ligand-activated ERβ in the presence of androgen-stimulated AR.

4. Stimulation of ERβ by its ligand results in widespread changes in AR-dependent gene expression.

5. Mechanistically, this effect appears to be mediated by competition between ERβ and AR for shared DNA-binding sites, which are highly enriched for ERβ, AR and FOXA1 motifs. These binding sites are shown to be highly transcriptionally active, with the result that ligand-activated ERβ reprograms a subset of androgen-dependent genes.

5.2 Results

5.2.1 ERβ expression in clinical datasets

Greater ERβ expression is associated with improved BCR-free survival

Firstly, to establish whether ERβ expression levels in prostate tissue correlate with clinical outcome, a large clinical dataset (CamCaP) [Ross-Adams et al., 2015] was interrogated using a published, online software tool [Dunning et al., 2017]. This dataset was generated by integration of copy number analysis and transcriptomics data from 482 tumour, benign and germline samples obtained from 259 men with primary prostate cancer. The integrated data were then used to identify five prognostic groups (iClusters) with distinct transcript expression profiles and genomic alterations.

ERβ expression was observed to vary significantly across the five prognostic groups (ANOVA $p = 0.04$) (Figure 5.2A). Interestingly, ERβ expression was highest in iClusters 2 and 4, which are associated with significantly greater BCR-free survival than iClusters 1 and 3 ($p = 0.017$ [Ross-Adams et al., 2015]). Furthermore, iClusters 2 and 4 feature fewer copy number abberations than the other prognostic groups. Taken together, these findings suggest that sustained ERβ expression in prostate cancer is associated with better clinical prognosis, potentially mediated through greater genome stability. This association appears to be independent of AR expression, which does not vary significantly across the iClusters (Figure 5.2B). However, it is interesting to note a trend towards lower AR expression in iClusters 2 and 4, where ERβ expression is greatest.

ERβ expression is inhibited by AR *in vivo*

Secondly, to investigate the relationship between ERβ and AR, gene expression data from two clinical datasets [Rajan et al., 2014; Shaw et al., 2016] were interrogated to determine the effect of AR inhibition (mediated by ADT) on ERβ expression. Rajan et al. (2014) performed RNA-seq on prostate tumours obtained from seven patients with locally advanced or metastatic disease before and after initiation of ADT (GSE48403). Similarly, Shaw et al., (2016) obtained

high-risk prostate cancer tissue from 27 patients treated with the LHRH antagonist degarelix and 20 matched, untreated controls (GSE72920). In both datasets, inhibition of AR signalling with treatment resulted in significant upregulation of ERβ mRNA expression ($p = 0.04$ and $p = 0.02$ respectively)(Figure 5.2C and D). These data suggest that ERβ expression is negatively regulated by AR in prostate cancer, a finding supported by previous observations showing that AR is more highly expressed in malignant than benign prostate epithelium [Ricciardelli et al., 2005]. Taken together, these observations may explain the decreased ERβ expression observed in malignant versus benign prostate tissue in previously published IHC studies [Asgari and Morakabati, 2011; Attia and Ederveen, 2012; Bonkhoff et al., 1999; Horvath et al., 2001].

5.2.2 ERβ expression in degarelix-treated prostate tissue

Tissue staining assessed by Dr. A.Y. Warren, Consultant Histopathologist, Cambridge University Hospitals NHS Foundation Trust.

To further investigate these findings, a TMA containing prostate biopsy cores from the 27 degarelix-treated patients and 20 matched, untreated controls [Shaw et al., 2016] was stained for ERβ using the validated CWK-F12 ERβ antibody. Staining of luminal epithelial cells was scored using the Allred system, which has been validated for assessment of ERα expression by IHC in breast cancer [Harvey et al., 1999] (Table 5.1). Strong ERβ staining was noted in basal epithelial cells of benign glands (Figure 5.3A), however these were not included in the analysis as firstly, they are not used in the Allred score and secondly, de-differentiation of basal epithelial cells is an early development in malignant transformation of prostate epithelium [Bonkhoff, 1996; Korshak et al., 2016]. They would therefore not be present in tumour samples to enable direct comparisons between benign and malignant regions.

ERβ staining in degarelix-treated and untreated controls was compared using a Wilcoxon rank sum test (Figure 5.3B). In both benign and tumour areas of prostate tissue, there was a trend towards increased ERβ expression following

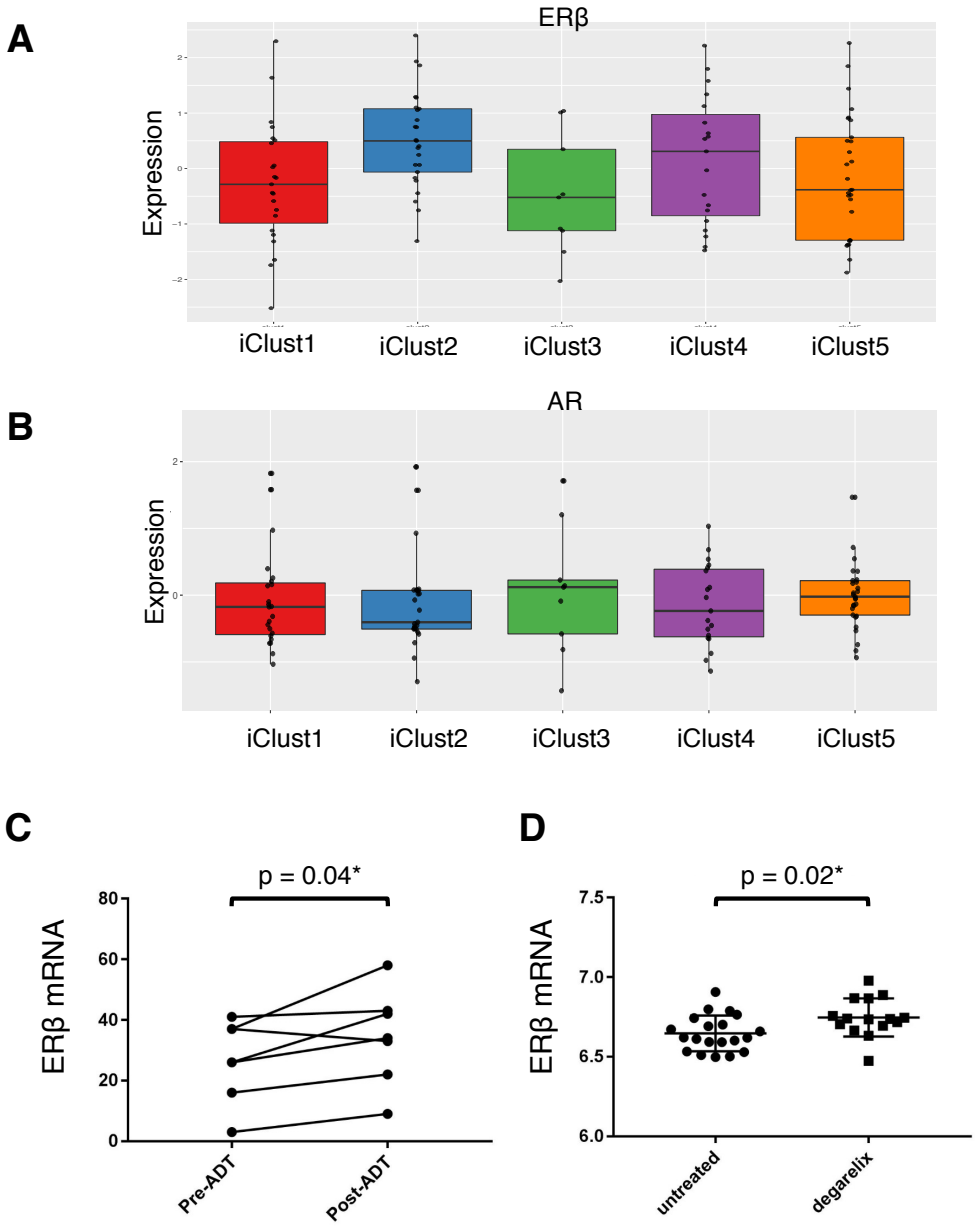


Figure 5.2: **ERβ expression in clinical datasets**. (A) ERβ gene expression in the CamCaP dataset is greatest in iClusters 2 and 4, which in turn are associated with greater BCR-free survival than iClusters 1 and 3. (B) There is no variability in AR expression across iClusters (p = 0.71). (C) ERβ mRNA expression data from matched tumour samples pre- and post-administration of ADT shows upregulation of ERβ expression in response to AR inhibition [Rajan et al., 2014]. (D) This was confirmed in a second cohort of patients treated with the LHRH antagonist degarelix [Shaw et al., 2016], suggesting that AR represses ERβ expression *in vivo*.

Proportion scoring (Percentage cells ER positive)	Staining intensity score
0 (0)	0 - Negative
1 (≤1)	1 - Weak
2 (1-10)	2 - Intermediate
3 (11-33)	3 - Strong
4 (34-66)	
5 (67-100)	

Table 5.1: **The Allred IHC Scoring System** was originally validated for ERα staining in breast cancer. The system combines the proportion of cells stained, and the staining intensity to stratify breast cancer according to the likelihood of disease response to hormonal therapy [Harvey et al., 1999].

degarelix treatment, although this was not statistically significant (p = 0.27 for benign tissue, p = 0.77 for tumour). This non-significance at a protein level (in contrast to the changes observed at a transcript level (Figure 5.2C and D)) may be due to the small sample size.

5.2.3 ERβ expression is inhibited by AR *in vitro*

In order to confirm regulation of ERβ expression by AR in an *in vitro* model, LNCaP cells were treated with siRNA to AR, and expression of ERβ mRNA quantified by RT-qPCR. Silencing of AR *in vitro* resulted in significant upregulation of ERβ expression (p = 0.03), confirming the *in vivo* data and further demonstrating that ERβ expression is inhibited by AR in malignant prostate epithelial cells (Figure 5.4).

The work presented thus far demonstrates that sustained ERβ expression in clinical samples is associated with improved BCR-free survival, and ERβ expression is inhibited by AR *in vitro* and *in vivo*. The decrease in ERβ expression previously observed in malignant transformation of the prostate [Asgari and Morakabati, 2011; Bonkhoff et al., 1999; Dey et al., 2014; Horvath et al., 2001; Leav et al., 2001] is therefore likely to be a direct consequence of the increase in AR signalling

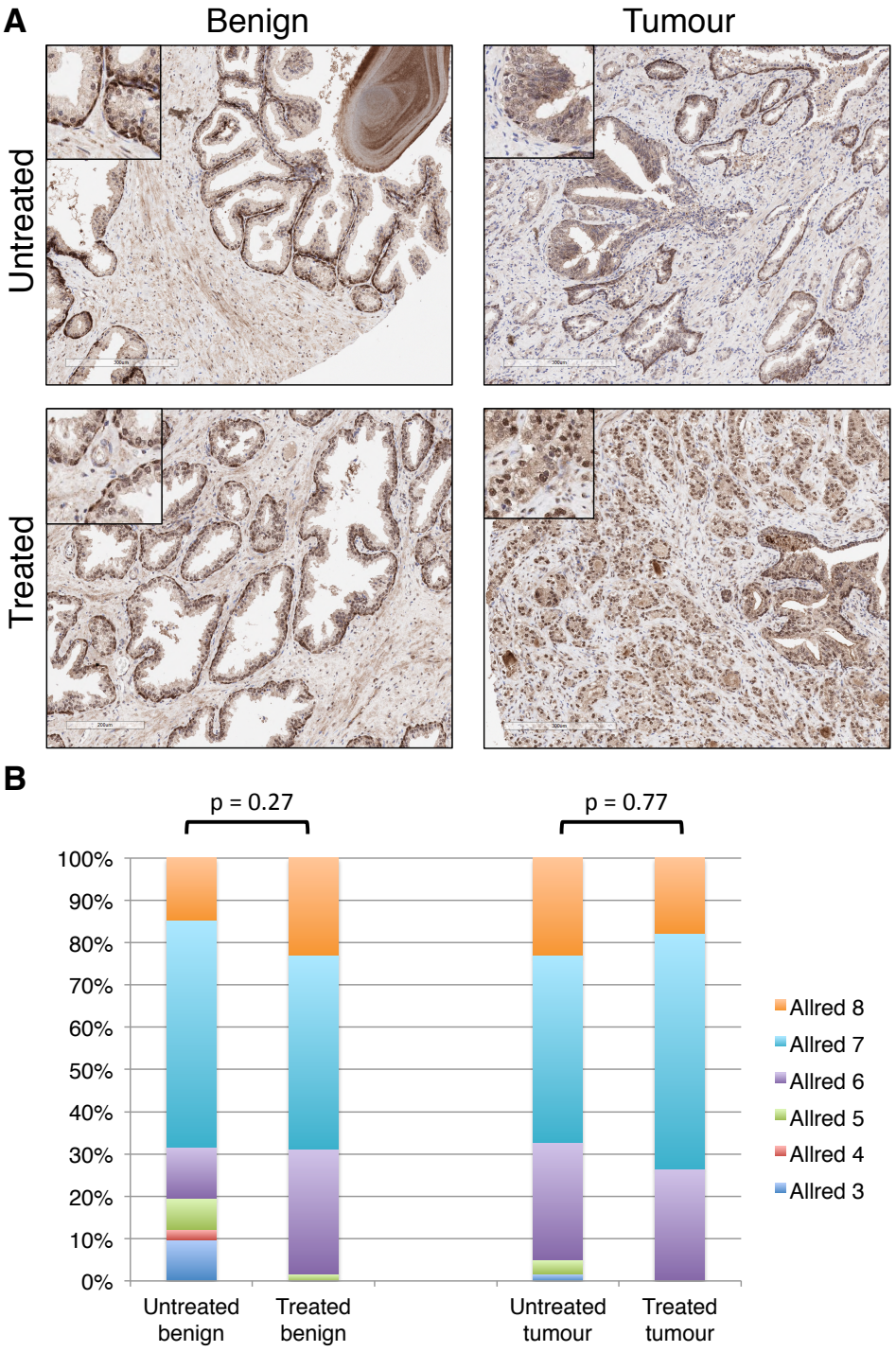


Figure 5.3: **ERβ expression in degarelix-treated prostate tissue.** (A) Strong ERβ expression is evident in the basal epithelial cells of benign glands. (B) Although not statistically significant, in both benign and tumour tissue there was a trend towards increased ERβ expression in luminal epithelial cells following degarelix treatment.

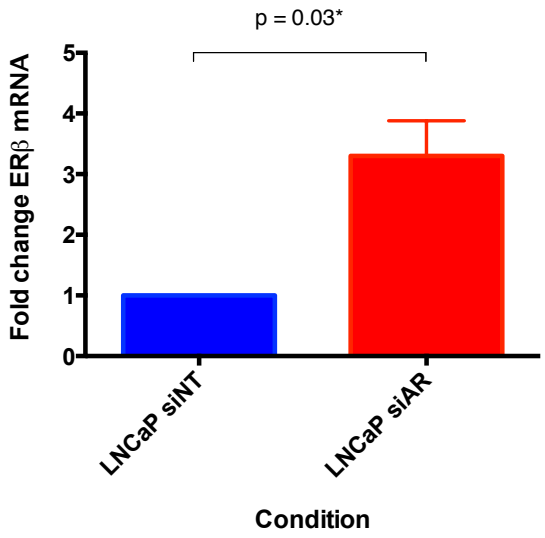


Figure 5.4: **ERβ expression is inhibited by AR *in vitro*.** 3.5-fold upregulation of ERβ expression occurred in response to AR knockdown in LNCaP cells.

that occurs in early prostate carcinogenesis [Mills, 2014; Zhou et al., 2015] (Section 5.1.1). We therefore wanted to investigate the effect of maintaining ERβ signalling in a prostate cancer *in vitro* model at phenotypic, transcriptomic and genomic levels, to determine if activation of ERβ can modulate this AR-driven prostate carcinogenesis. The doxycycline-inducible LNCaP-ERβ cell line (Chapter 4) was therefore used in subsequent experiments.

5.2.4 Ligand-activated ERβ inhibits prostate cancer cell proliferation

To investigate the effect of ERβ signalling on prostate cancer cell growth, LNCaP-ERβ+ cells (ERβ expression induced with 0.1 μg/ml doxycycline for 24 hours) and LNCaP-ERβ- cells were treated with vehicle, 1 nM R1881, 10 nM 3β-adiol or both R1881 and 3β-adiol following 3 days of hormone deprivation. R1881 is a synthetic, non-aromatisable androgen, which binds to and stimulates AR (National Center for Biotechnology Information. PubChem Compound Database; CID=261000, https://pubchem.ncbi.nlm.nih.gov/compound/261000 (accessed Sept.

15, 2017).). As it is non-aromatisable and therefore not converted to estrogenic metabolites, potential cross-ligand activation of ERβ is avoided. 3β-adiol is an ERβ-selective DHT metabolite thought to be the natural ligand for ERβ [Oliveira et al., 2007; Weihua et al., 2002a,b]. It is found in high concentration in benign prostatic tissue [Piccolella et al., 2014]. Cell proliferation was recorded for 7 days following treatment (Figure 5.5).

The androgen-responsive nature of the LNCaP-ERβ cell line was confirmed by treating LNCaP-ERβ- cells with R1881, which resulted in increased cell proliferation as compared with vehicle (Figure 5.5A). Interestingly, 3β-adiol treatment in LNCaP-ERβ- cells resulted in a similar increase in cell proliferation. This may be in keeping with previous reports showing that 3β-adiol can stimulate AR in testosterone-deplete conditions [Mizokami et al., 2004].

LNCaP-ERβ+ cells treated with both R1881 and 3β-adiol showed significant decrease in cell proliferation compared firstly, to vehicle-treated cells ($p = 0.006$, figure 5.5B) and secondly, to LNCaP-ERβ- cells treated with R1881 and 3β-adiol ($p = 0.02$, figure 5.5F). Cell morphology did not change appreciably with any of the treatment conditions (Figure 5.6). Intriguingly, in this experiment, cell proliferation was only inhibited when i) ERβ is expressed; ii) ERβ is activated by 3β-adiol and iii) AR is stimulated by androgen. This suggested that the mechanism of growth inhibition could be related to competitive antagonism between the two nuclear receptors. Interestingly, this mirrors work previously published from the Carroll laboratory, showing that in breast cancer, progesterone is only anti-proliferative when progesterone receptor (PR) is expressed in the presence of estrogen-stimulated ERα [Mohammed et al., 2015].

5.2.5 Preliminary ERβ ChIP-seq to determine duration of 3β-adiol treatment

Bioinformatic analysis of ChIP-seq data by Dr. I. Chernukhin, CRUK Cambridge Institute

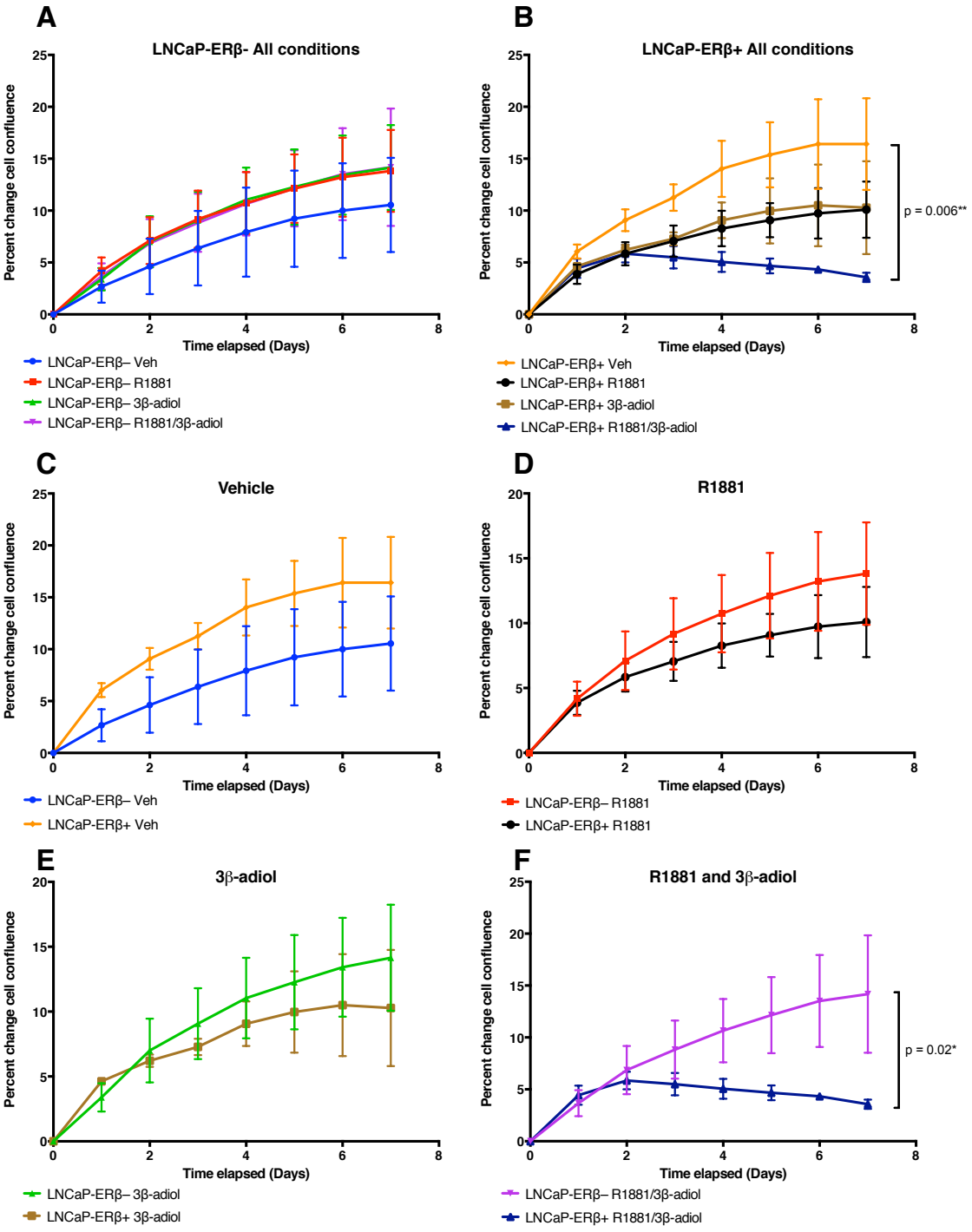


Figure 5.5: **Ligand-activated ERβ inhibits prostate cancer cell growth in the presence of androgen-stimulated AR.** (A) LNCaP-ERβ- cells retain androgen responsiveness, as indicated by an increase in proliferation with R1881 treatment. (B) The presence of ERβ alone did not significantly alter proliferation (c.f panel C), only with R1881 and 3β-adiol treatment was there any significant change compared to vehicle ($p = 0.006$). (F) The inhibitory effect of R1881 and 3β-adiol combined treatment is mediated by the presence of ERβ.

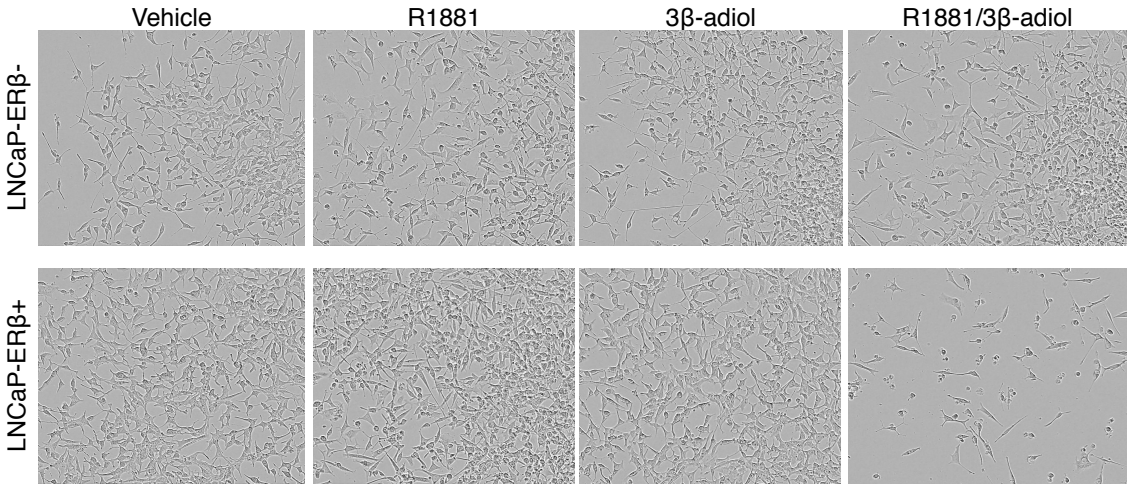


Figure 5.6: **LNCaP-ERβ cell morphology did not change under treatment conditions,** suggesting that the differences in cell confluence detected by the IncuCyte™ were due to inhibition of cell proliferation, rather than cytotoxicity.

To investigate the mechanisms of these observed effects on cell growth at genomic and transcriptomic levels it was necessary to determine the optimum duration of hormone treatment for the LNCaP-ERβ cells, prior to conducting large-scale RNA-seq and ChIP-seq experiments. Work previously published from the laboratory suggested that 4 hours was the optimum duration of R1881 treatment [Robinson et al., 2011], however the optimum duration of 3β-adiol treatment was not clear from previously published literature.

LNCaP-ERβ+ cells were treated with 3β-adiol or vehicle for 1, 4 or 24 hours prior to harvesting for ChIP-seq. Two of the validated ERβ antibodies (MC10 and CWK-F12), which are both monoclonal, were pooled in equal quantity to be used for the immunoprecipitation. As these two antibodies bind to different epitopes (Figure 2.1), we felt that this approach would increase the efficiency of the IP by creating a 'polyclonal-like' effect. Average numbers of ERβ peaks called in each condition are displayed in table 5.2. As the four-hour time point generated both the greatest differential between vehicle and 3β-adiol conditions, and the greatest number of peaks altogether, this was used for subsequent analysis and experiments.

Time (hrs)	Treatment	No. Peaks
1	Vehicle	15,132
1	3β-adiol	15,057
4	Vehicle	16,182
4	3β-adiol	18,899
24	Vehicle	16,216
24	3β-adiol	17,600

Table 5.2: **Number of ERβ peaks called with 1, 4 or 24 hour 3β-adiol treatments.**

The distribution of ERβ binding sites across genomic features is shown in figure 5.7A. Genome-wide ERβ binding sites were subsequently overlapped with previously published AR and FOXA1 cistromes from LNCaP cells [Robinson et al., 2011] (Figure 5.7B). Interestingly, this revealed 3,954 sites shared between ERβ and AR, and 2,906 sites shared between ERβ, AR and FOXA1. This, to our knowledge is the first genome-wide data in any cell- or tissue-context to suggest that ERβ and AR share DNA-binding sites. This finding provided direct evidence of nuclear receptor crosstalk at a genomic level, and an early indication that ERβ may indeed be able to influence AR function in prostate cancer through competition for DNA-binding sites. This in turn would begin to explain the observed inhibition of cell proliferation in LNCaP-ERβ+ cells treated with R1881 and 3β-adiol (Figure 5.5B and F).

5.2.6 Ligand-activated ERβ alters the AR transcriptome in prostate cancer

5.2.6.1 Experimental design

In order to further investigate the effects of ERβ on AR function a large combined RNA-seq and ChIP-seq experiment was performed. LNCaP-ERβ cells were cultured, treated and harvested simultaneously for each of the 4 replicates of ChIP-seq and 4 of the RNA-seq replicates in order to obtain matched datasets.

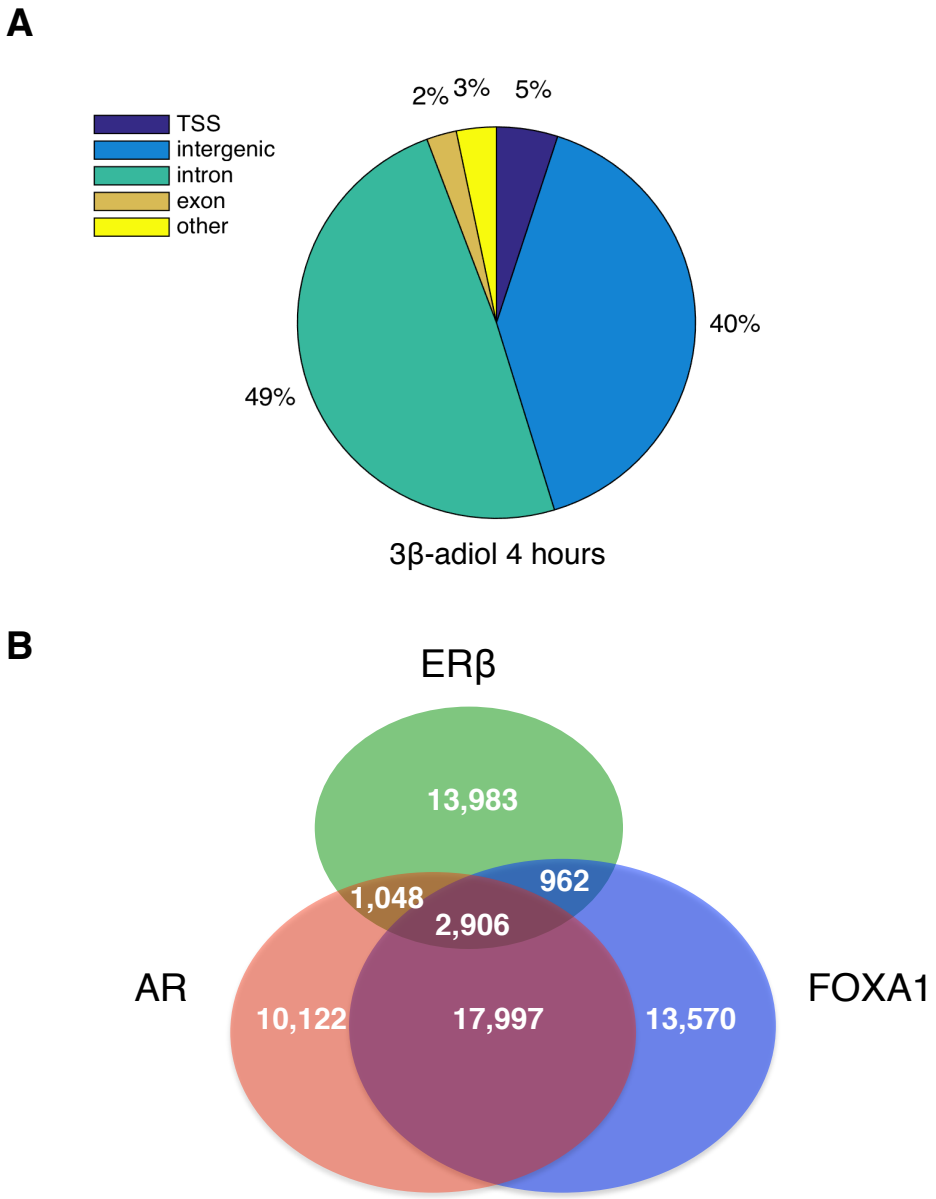


Figure 5.7: **Analysis of ERβ binding following 4 hour treatment with 3β-adiol** (A) Distribution of ERβ DNA binding sites across genomic features. (B) Genome-wide ERβ binding in the 3β-adiol-treated LNCaP-ERβ+ cells was overlapped with AR and FOXA1 cistromes generated in LNCaP cells [Robinson et al., 2011]. This revealed 3,954 sites shared between ERβ and AR, and 2,906 sites shared between ERβ, AR and FOXA1.

A further 2 replicates (to make a total of 6) were performed for RNA-seq. All replicates were within 2 cell passages of the preceding replicate. Serum-starved LNCaP-ERβ- cells and LNCaP-ERβ+ cells were treated with either vehicle, 1 nM R1881, 10 nM 3β-adiol or both R1881 and 3β-adiol for 4 hours (Table 5.3). Differential gene expression for each pair-wise comparison (total of 28 comparisons) was generated in order that all control and test conditions could be contained within a single experiment. This approach enabled detailed study of the effects of each individual variable within the model.

Cell line	LNCaP-ERβ–	LNCaP-ERβ+
Condition	Vehicle	Vehicle
	R1881	R1881
	3β-adiol	3β-adiol
	R1881 + 3β-adiol	R1881 + 3β-adiol

Table 5.3: **Experimental design for ERβ/AR crosstalk experiment.** Matched RNA-seq and ERβ ChIP-seq was performed according to these conditions. By including all combinations of the conditions, it was possible to control for the effects of each variable (i.e. each receptor +/- ligand) within a single experiment.

5.2.6.2 RNA-sequencing - Key controls

In the absence of ERβ expression, LNCaP-ERβ- cells remain androgen-responsive. In this positive control, we observed that treating LNCaP-ERβ- cells with 1 nM R1881 resulted in significant changes in gene expression as compared with vehicle treatment (115 genes, FDR <0.05). By way of validation, many known AR target genes were observed in this group, for example KLK3, HOXB13, KLK2, CAMKK2 and FKPB5 (Figure 5.8A and B).

In the absence of ERβ expression, 3β-adiol does not alter R1881-mediated gene expression. There were no significant changes in gene expression (0 genes, FDR <0.05) when comparing R1881 alone versus R1881 and

3β-adiol in LNCaP-ERβ- cells, indicating that 3β-adiol does not significantly cross-react with AR in the presence of androgen (Figure 5.8C and D).

Unliganded ERβ only produces minor changes in R1881-mediated gene expression. Thirty-five genes (FDR <0.05) were differentially expressed in R1881-treated LNCaP-ERβ+ cells versus R1881-treated LNCaP-ERβ- cells (Figure 5.8E and F). This suggests that in this system ERβ may have some constitutive activity, however major changes in gene expression are only observed when ERβ is ligand-activated.

5.2.6.3 Ligand-activated ERβ downregulates AR target genes

Profound differences in gene expression were observed comparing R1881-treated LNCaP-ERβ- cells *versus* LNCaP-ERβ+ cells treated with R1881 and 3β-adiol (Figure 5.9). 1,312 genes were differentially expressed (FDR <0.05). This important result showed that ligand-activated ERβ, in androgen replete conditions produces widespread and significant changes in gene expression in a prostate cancer cell line, reflecting the previously observed changes in cell proliferation (Figure 5.5B and F).

To further interrogate the effects of ligand-activated ERβ on AR-driven gene expression, a set of 115 androgen-dependent genes was identified from the positive control (LNCaP-ERβ- with vehicle *versus* LNCaP-ERβ- with R1881)(Figure 5.8A). The effects of 3β-adiol-stimulated ERβ on the expression of genes within this set were then determined (Figure 5.10A). Seventy percent of the 115 genes upregulated by R1881 in LNCaP-ERβ- cells were subsequently downregulated in the presence of ligand-activated ERβ, with an average 1.7-fold decrease in expression ($p < 0.0001$). Interestingly, the expression of AR mRNA itself was seen to decrease significantly ($p = 0.002$) in the presence of ligand-activated ERβ (Figure 5.10B), suggesting that the mechanism of this effect may be through direct downregulation of AR expression by ERβ.

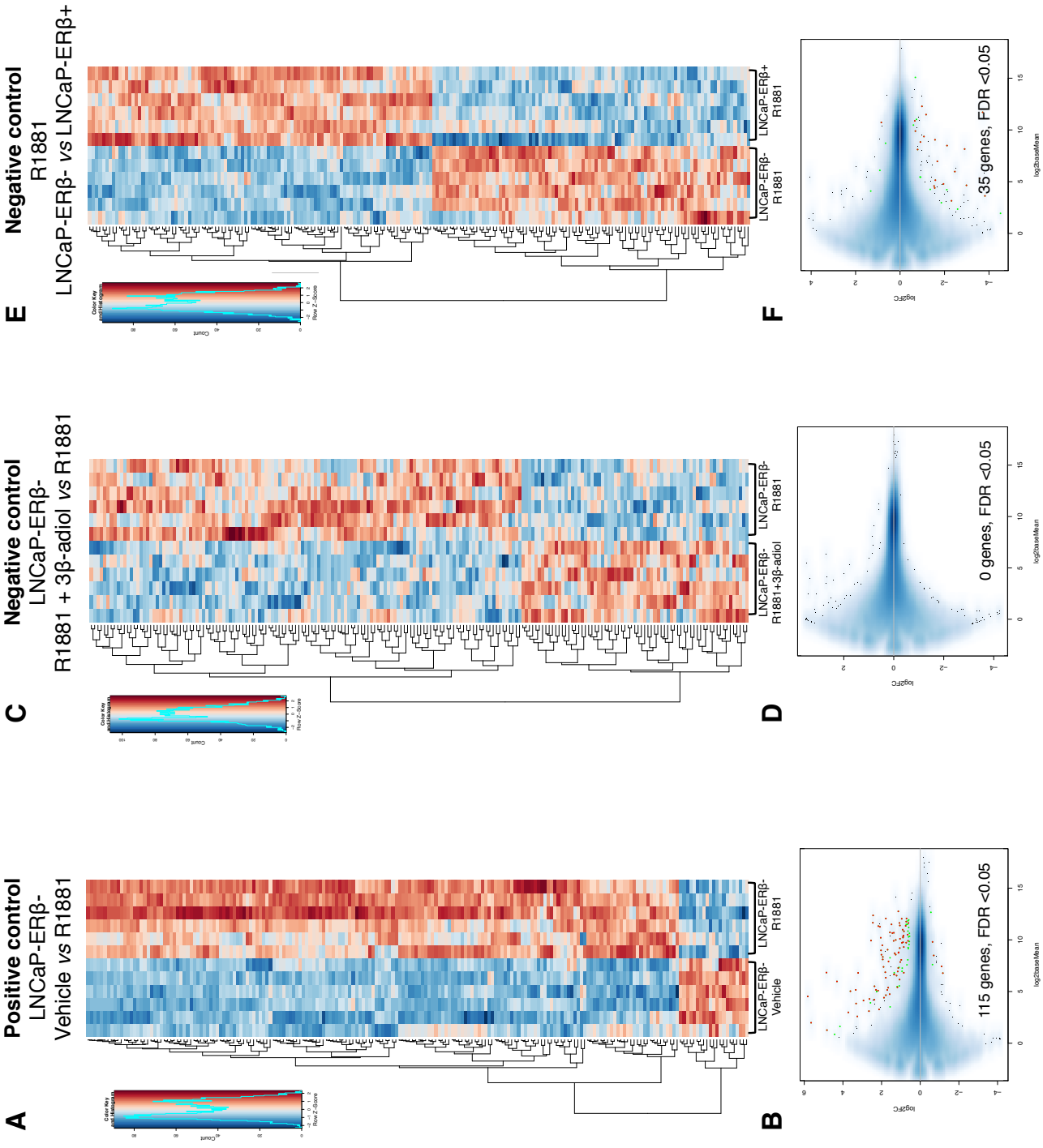


Figure 5.8: **Key controls in ERβ/AR crosstalk RNA-seq** (A) LNCaP-ERβ- cells remain androgen-responsive, with (B) 115 genes differentially regulated (FDR <0.05) by R1881. (C, D) Without ERβ, 3β-adiol does not significantly alter R1881-mediated gene expression. (E) Unliganded ERβ only produces minor changes in R1881-mediated gene expression, with (F) 35 differentially expressed genes (FDR <0.05). Heatmaps show top 200 differentially expressed genes.

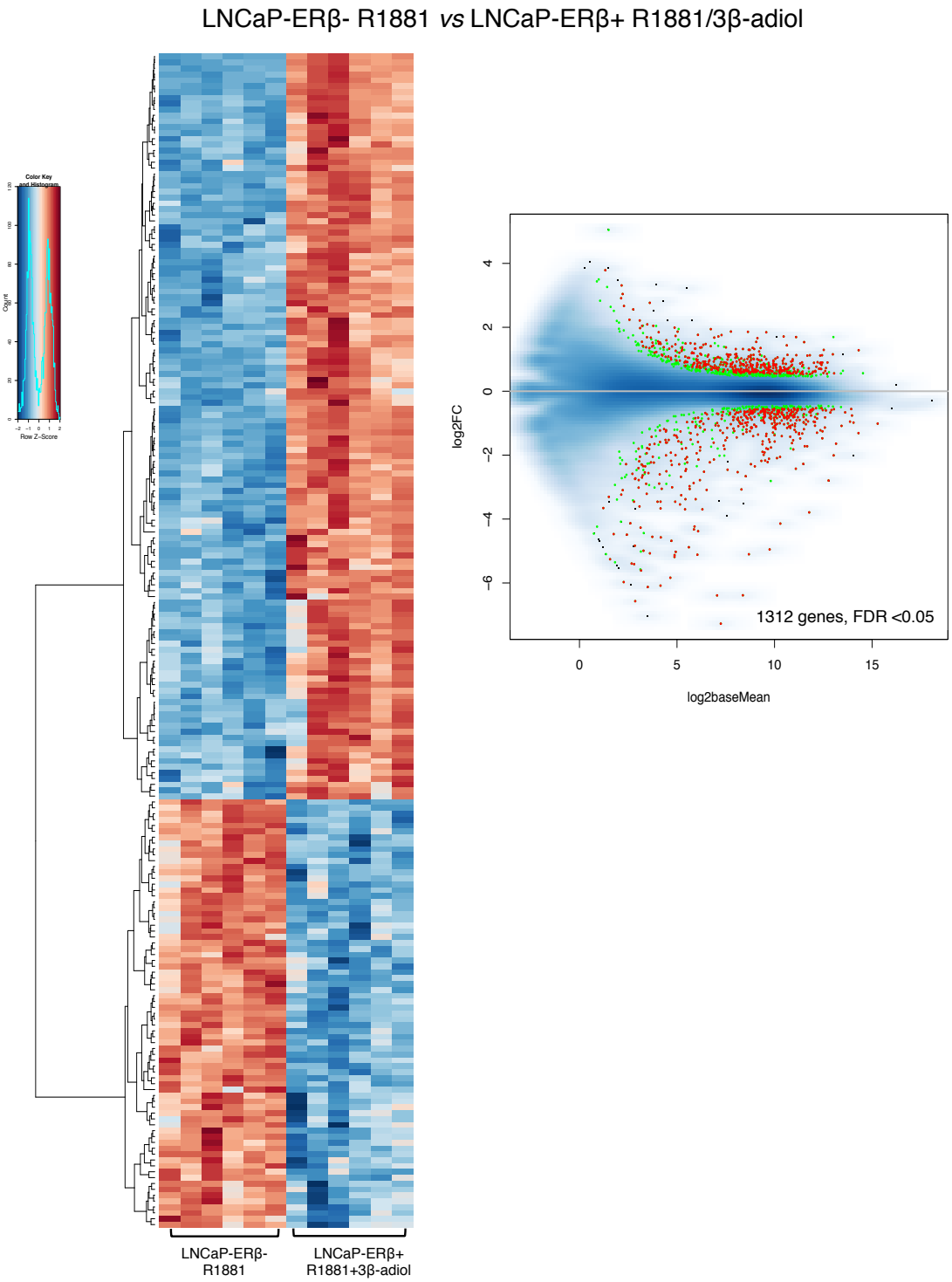


Figure 5.9: **Ligand-activated ERβ produces widespread change in gene expression in R1881-treated prostate cancer cells.** Introducing 3β-adiol-stimulated ERβ into androgen-stimulated LNCaP cells resulted in significant differential expression of 1,312 genes (FDR <0.05). Heatmap shows top 200 differentially expressed genes.

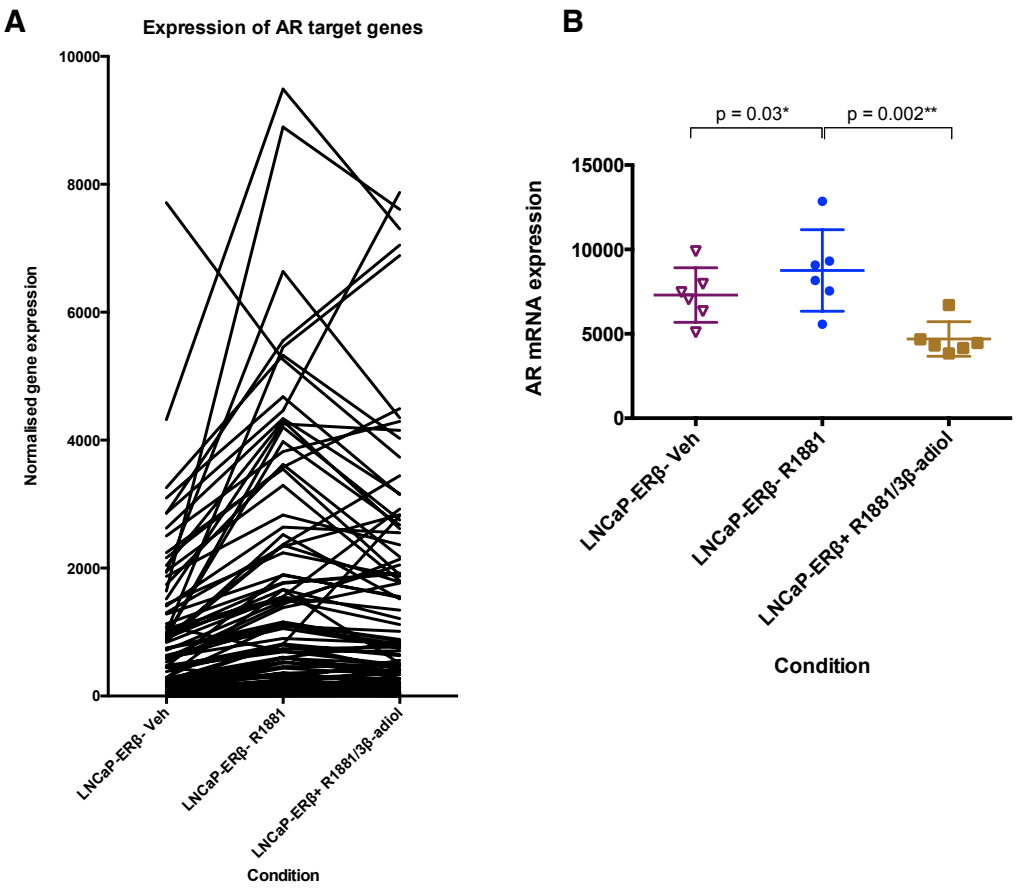


Figure 5.10: **Ligand-activated ERβ downregulates AR-dependent genes.** (A) Of the 115 AR-dependent genes identified, 70% were significantly downregulated by ligand-activated ERβ. (B) Expression of AR mRNA was significantly decreased by ERβ, suggesting that ERβ can directly suppress AR expression.

5.2.7 ERβ competes with AR for shared DNA-binding sites to influence AR-dependent gene expression

Bioinformatic analysis of ChIP-seq, and integration with RNA-seq data by Dr. I. Chernukhin, CRUK Cambridge Institute

Having demonstrated that ligand-stimulated ERβ downregulates AR-dependent genes and decreases androgen-mediated cell proliferation, we wanted to determine the genomic mechanisms by which ERβ and AR were influencing one another.

Under experimental conditions matched to the RNA-seq experiment, ERβ and AR ChIP-seq were performed to map the genome-wide DNA binding of these receptors in the LNCaP-ERβ+ cell line model and determine the influence of the hormone treatments (R1881 alone, 3β-adiol alone or both R1881 and 3β-adiol) on their DNA-binding. As a control, ERβ ChIP-seq was also performed in LNCaP-ERβ- cells. Importantly, there was a negligible number of ERβ peaks identified in the LNCaP-ERβ- cells, further confirming the integrity of the experimental model, this time at a genomic level (Table 5.4). Unfortunately the AR ChIPs were unsuccessful, probably due to a failed batch of antibody, as further attempts to use this antibody by ChIP-qPCR were also not successful (data not shown). We are aware that other laboratories have simultaneously had similar difficulties with failed batches of this particular AR antibody (Prof. W.D. Tilley, University of Adelaide, personal communication).

In this cell line model, unliganded ERβ appears to have some constitutive binding as 13,800 peaks were called in the vehicle-treated condition. Treatment with 3β-adiol alone results in a strong increase in the number of peaks (17,327) and motifs (21,748) identified. Interestingly, it appears that the addition of R1881 in both 3β-adiol- and vehicle-treated conditions results in a decrease in the numbers of ERβ peaks (14,190 and 10,643 respectively) and motifs (18,130 and 15,075 respectively)(Table 5.4). In all conditions, ERβ peaks were highly enriched for consensus ERβ motifs ($p < 0.001$)(Figure 5.11).

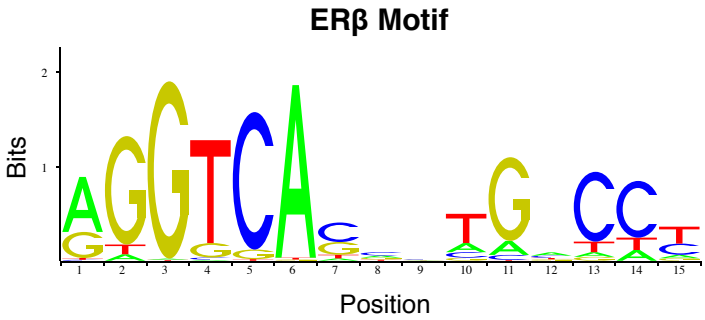


Figure 5.11: **ERβ peaks are significantly enriched for the ERβ motif** ($p < 0.001$).

Sample	Number ERβ peaks	Number ERβ Motifs	Peaks with ERβ motif (%)
LNCaP-ERβ- Veh	3	-	
LNCaP-ERβ- 3β-adiol	3	-	
LNCaP-ERβ- R1881	5	-	
LNCaP-ERβ- R1881+3β-adiol	0	-	
LNCaP-ERβ+ Veh	13800	18844	94
LNCaP-ERβ+ 3β-adiol	17327	21748	90
LNCaP-ERβ+ R1881	10643	15075	96
LNCaP-ERβ+ R1881+3β-adiol	14190	18130	91

Table 5.4: **ERβ peaks and motifs called by ChIP-seq under the experimental conditions tested.** The absence of ERβ peaks called in LNCaP-ERβ- cells confirms that ERβ is not expressed in the uninduced ERβ- condition. R1881 appears to decrease the number of ERβ peaks and motifs called in both vehicle and 3β-adiol-stimulated conditions.

These differences in absolute numbers of peaks and motifs called were further reflected in the variability of signal intensities detected at ERβ binding sites. Tag densities were measured in a +/- 2.5 kBp window, centred in the middle of peak regions detected in each experimental condition (Figure 5.12A-D). With respect to the peak intensities detected in each experimental condition, the pattern of variability in intensity with hormonal treatment is consistent. The highest intensity binding is in the 3β-adiol only treatment, followed by R1881 with 3β-adiol, followed by vehicle and finally, R1881 alone resulted in the lowest signal intensity. Taken together, these data suggest that that R1881-stimulated AR decreases ERβ binding in both the presence and absence of 3β-adiol and furthermore, decreases the signal intensity of conserved ERβ binding sites. This observation was partly reflected in the RNA-seq data, where we observed that R1881 stimulation in the absence of 3β-adiol resulted in differential expression of 94 genes (FDR <0.05)

in LNCaP-ERβ+ cells (comparing LNCaP-ERβ+ with vehicle *versus* LNCaP-ERβ+ with R1881)(Figure 5.13A). The same however was not true in the presence of 3β-adiol (comparing LNCaP-ERβ+ with 3β-adiol *versus* LNCaP-ERβ+ with R1881 and 3β-adiol), where no change in gene expression was detected (Figure 5.13B). The mechanism as to how R1881-stimulated AR represses ERβ binding in this model system is not presently clear. As the stably-transfected ERβ transgene is not in its endogenous locus, these data would suggest that AR is influencing ERβ mRNA or protein stability, rather than acting at the *ESR2* gene locus itself. Further study (in particular repeating the AR ChIP-seq intended as part of this experiment) is required to understand the details of these particular observations.

Distribution of DNA motifs across unique and shared binding sites

In view of two important factors; firstly, that LNCaP-ERβ+ cells treated with both R1881 and 3β-adiol showed significant changes in proliferation and gene expression and secondly, that the dual-hormone treatment is likely to be most physiologically relevant to early-stage prostate cancer (i.e. androgen replete conditions), further analyses focused on data obtained from LNCaP-ERβ+ cells treated with both R1881 and 3β-adiol.

DNA-binding sites shared between ERβ, AR and FOXA1 were identified by overlapping the ERβ cistrome from dual-hormone treated LNCaP-ERβ+ cells with AR and FOXA1 binding sites obtained from R1881-stimulated LNCaP cells [Robinson et al., 2011]. Under these treatment conditions, ~14,200 ERβ binding sites were identified, of which 3,480 were shared with AR (Figure 5.14A). Tag densities measured within a +/- 2.5 kBp window (Figure 5.12) demonstrate the precise co-localisation of the shared ERβ, AR and FOXA1 binding sites.

Motif analysis confirmed significant enrichment for ERβ, AR and FOXA1 consensus motifs within these ERβ/AR shared binding sites ($p < 0.001$)(Figure 5.14B). FOXA1 is known to be an important nuclear receptor co-factor for AR [Robinson et al., 2011, 2014], so the presence of the motif in the shared binding sites

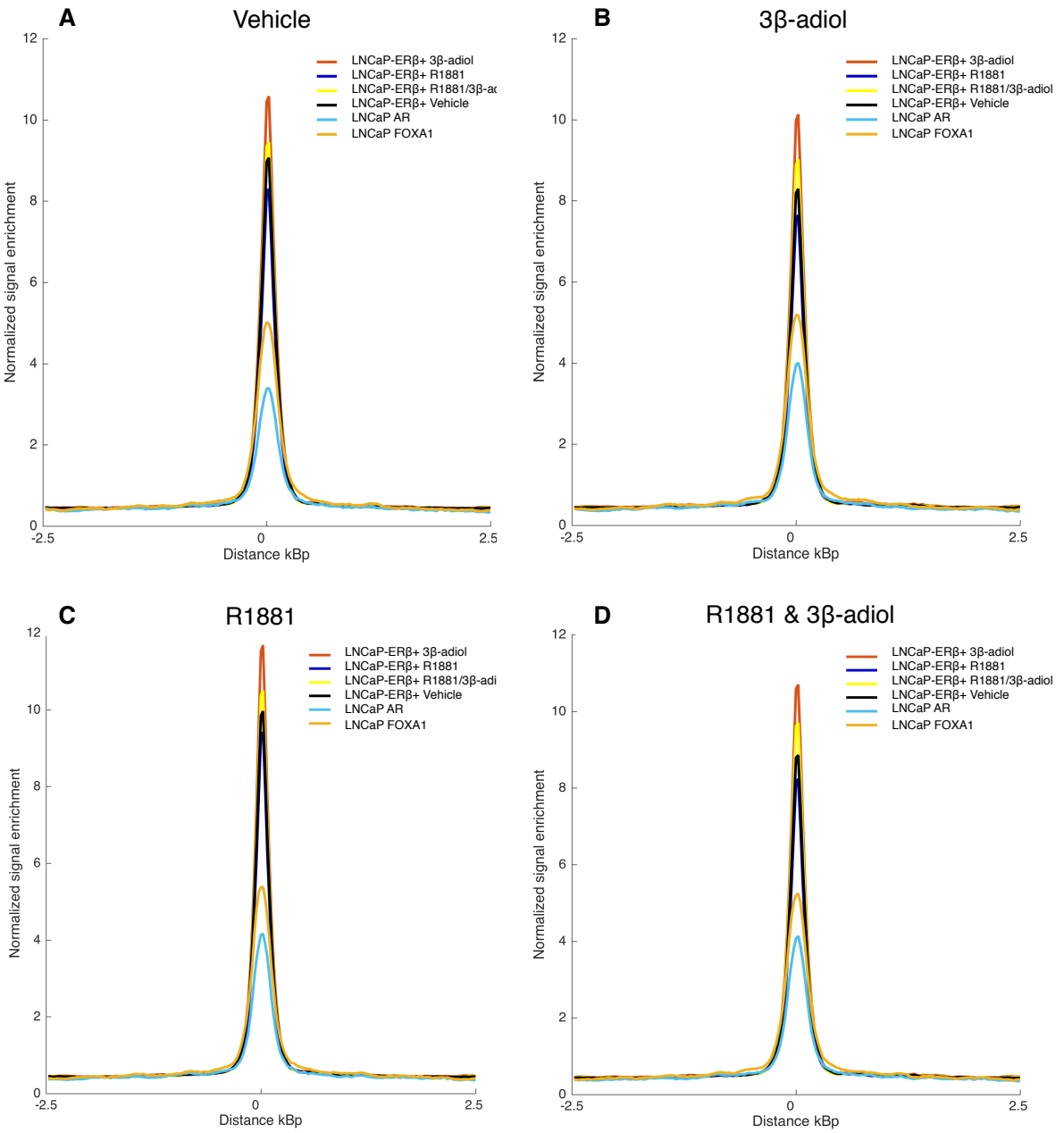


Figure 5.12: **ERβ ChIP-seq tag intensities,** centred in the middle of peak regions detected per experimental condition (A) vehicle; (B) 3β-adiol alone; (C) R1881 alone; (D) R1881 and 3β-adiol. In both the presence and absence of 3β-adiol, R1881 stimulation decreased the binding intensity of ERβ. ERβ binding sites were overlapped with AR and FOXA1 sites from LNCaP cells, [Robinson et al., 2011] revealing the precise co-localisation of ERβ/AR/FOXA1 binding at these sites.

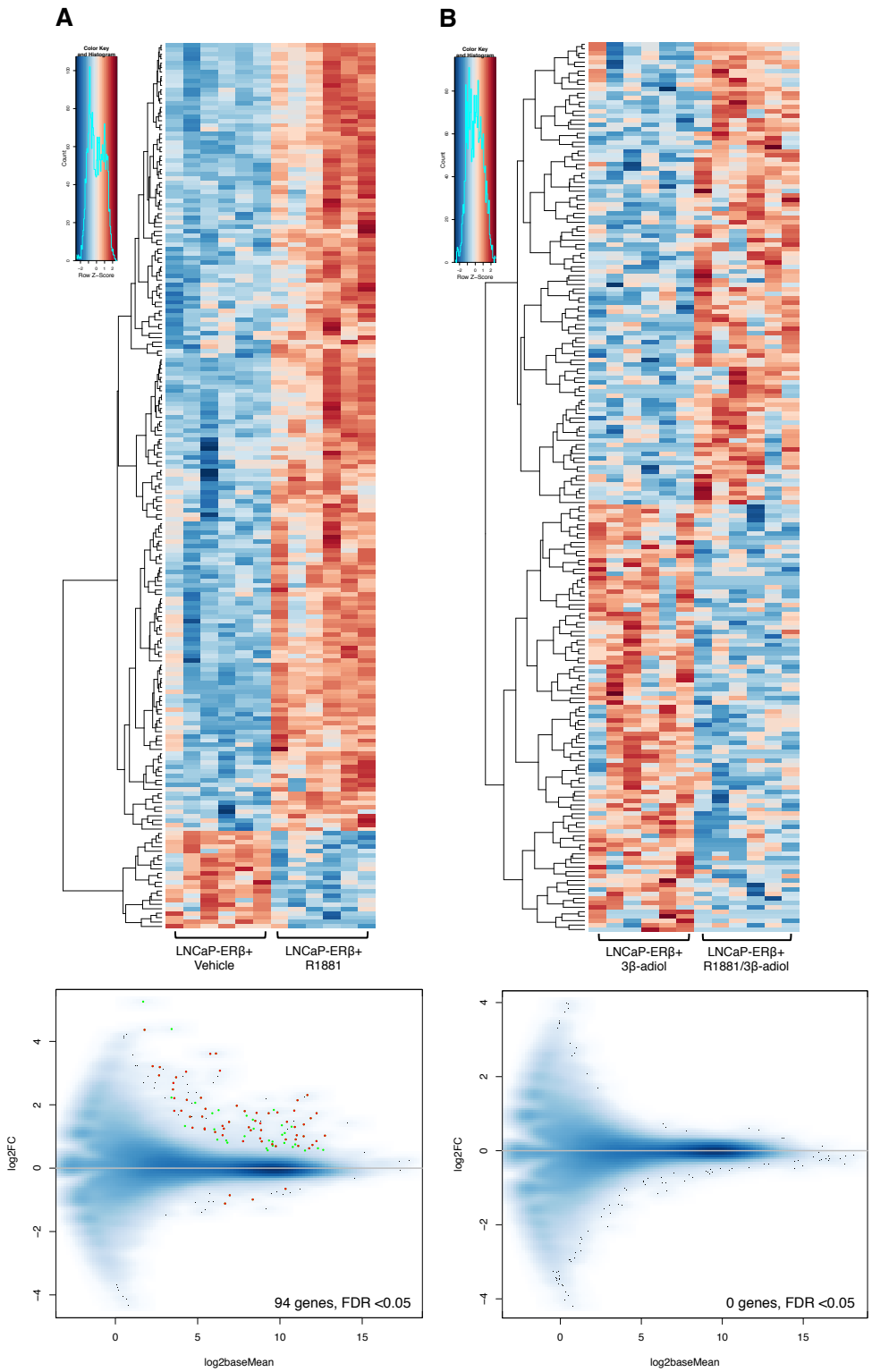


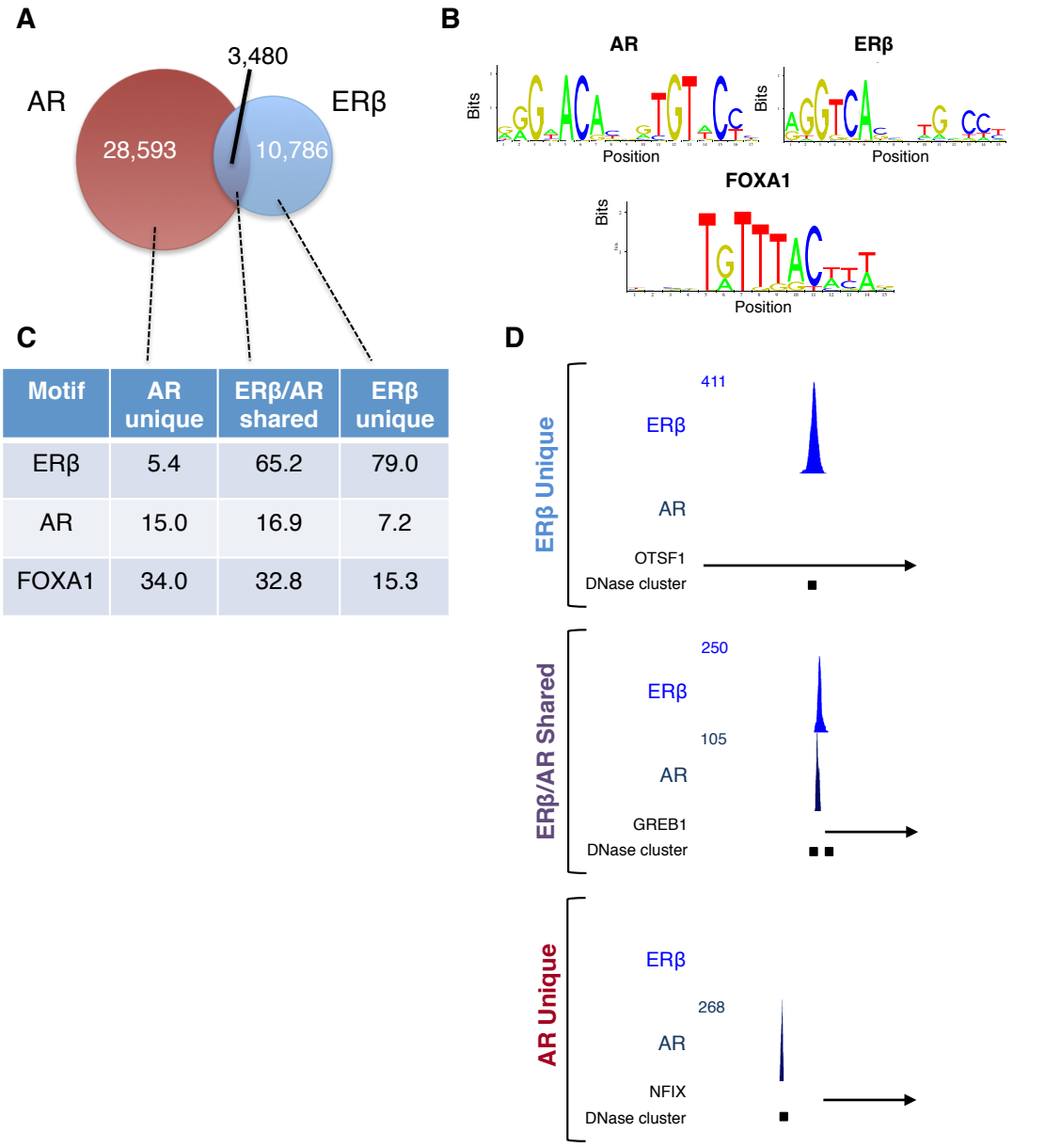
Figure 5.13: **AR-mediated changes in gene expression.** (A) In LNCaP-ERβ+ cells, R1881 results in altered expression of 94 genes (FDR <0.05), whereas when ERβ is stimulated by 3β-adiol (B), no such response is seen. Thus, in the presence of ERβ, R1881-stimulated AR only produces changes in gene expression when ERβ is unliganded. Heatmaps show top 200 differentially expressed genes.

was to be expected. Surprisingly however, the ERβ-unique binding sites were also significantly enriched for FOXA1 motifs ($p < 0.001$), suggesting that FOXA1 may serve as a co-factor to ERβ. To our knowledge, no association between ERβ and FOXA1 has been described in the literature. Further study is necessary to confirm a functional interaction between these factors.

To further understand the mechanisms governing the distribution of ERβ and AR binding across the shared and unique sites, we determined the percentage of AR-unique, ERβ/AR shared and ERβ-unique binding sites that contained ERβ and/or AR motifs (Figure 5.14C). The distribution of ERβ and AR motifs under peaks between the shared and unique sites indicates that the binding patterns observed are sequence-driven, i.e. the proportion of AR peaks with AR motifs was similar between the shared and AR-unique sites; the proportion of ERβ peaks with ERβ motifs was similar between the shared and ERβ-unique sites but the proportion of AR-unique sites with ERβ motifs was very low and *vice versa*. Examples of ERβ and AR peaks called from shared and unique sites are shown in figure 5.14D. The percentage of ERβ-unique binding regions containing FOXA1 motifs further suggests a role for this co-factor in the genomic function of ERβ (Figure 5.14C).

Shared ERβ/AR binding sites are highly transcriptionally active

Integration of the ChIP-seq and RNA-seq data demonstrates that the 3,480 shared binding sites are more transcriptionally active than ERβ-unique or AR-unique binding sites (Figure 5.15). We used hierarchical clustering to rank expression of androgen-regulated genes within 50 kb of identified DNA-binding sites. Treatment with both R1881 and 3β-adiol results in an alternate pattern of gene expression from the shared binding sites as compared with either hormone alone, or either ERβ- or AR-unique binding sites. This suggests that in the presence of R1881-stimulated AR, ligand-activated ERβ reprograms androgen-dependent gene expression, potentially by competing for these shared binding sites, or possibly by sequestration of available co-factors such as FOXA1. Further work is required to understand these mechanisms in more detail.



Motif	AR unique	ERβ/AR shared	ERβ unique
ERβ	5.4	65.2	79.0
AR	15.0	16.9	7.2
FOXA1	34.0	32.8	15.3

Figure 5.14: **ChIP-seq reveals shared ERβ/AR binding sites.** (A) ERβ binding sites from LNCaP-ERβ+ cells treated with R1881 and 3β-adiol were overlapped with AR binding sites from R1881-stimulated LNCaP [Robinson et al., 2011], showing 3,480 shared binding sites. (B) Shared ERβ/AR binding sites were significantly enriched (p <0.001) for AR, ERβ and FOXA1 motifs. Significant enrichment for FOXA1 in ERβ-unique sites (p <0.001) suggests a co-factor role for FOXA1 with respect to ERβ. (C) The percentage of AR-unique, ERβ/AR shared and ERβ-unique binding sites containing ERβ, AR and FOXA1 motifs is shown, suggesting that the distribution of ERβ/AR binding is sequence driven with a potential role for FOXA1 in ERβ binding. (D) Examples of ERβ and AR binding at shared and unique sites. DNase cluster data from UCSC genome browser.

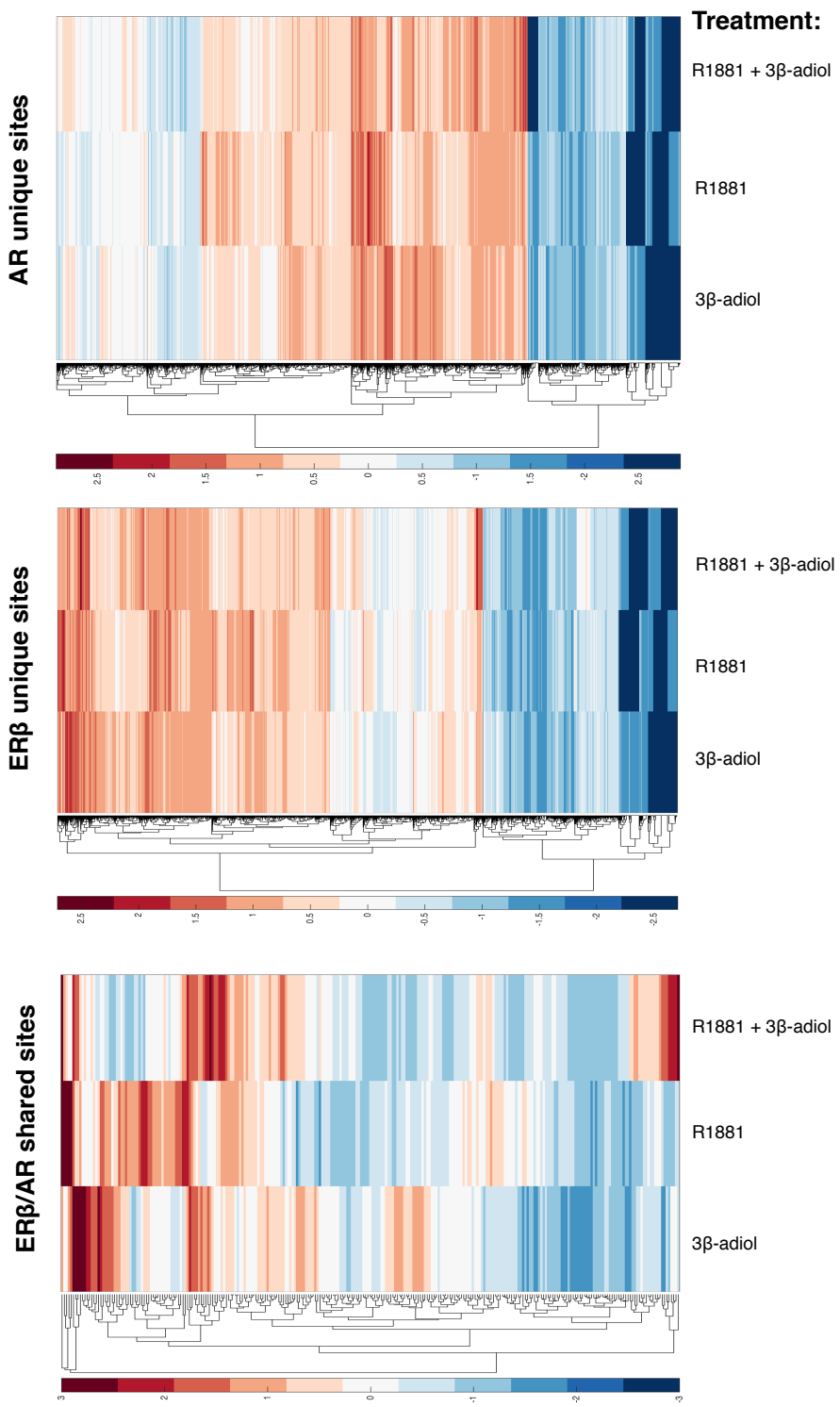


Figure 5.15: **Integration of ChIP- and RNA-seq reveals that shared ERβ/AR binding sites are highly transcriptionally active.** Furthermore, under dual hormone treatment an alternate transcriptional profile is apparent from shared sites, suggesting that ERβ reprograms AR-dependent gene expression. Androgen-regulated genes within 50 kb of binding sites are ranked by hierarchical clustering under different treatment conditions.

5.3 Summary

On the basis of previously published studies suggesting that i) ERβ is a tumour suppressor lost in malignant transformation of the prostate [Asgari and Morakabati, 2011; Attia and Ederveen, 2012; Dey et al., 2014; Horvath et al., 2001; Leav et al., 2001; Risbridger et al., 2007] and ii) ERβ and AR influence each other through nuclear receptor crosstalk [Grubisha and DeFranco, 2013; Mizokami et al., 2004; Rizza et al., 2014; Teng et al., 2014; Thelen et al., 2007; Weng et al., 2013], a number of studies were undertaken to test the hypothesis that ERβ modulates AR-driven prostate carcinogenesis. The aim of these studies was to investigate the relationship between ERβ and AR in both *in vitro* and *in vivo* contexts and to explore whether a selective ERβ agonist could be used therapeutically to modify early prostate cancer progression.

A large clinical dataset integrating gene expression data and copy number aberrations was examined for correlation between ERβ expression and clinical outcome. This revealed that greater ERβ expression was associated with improved BCR-free survival. This association was observed to be independent of AR expression, which did not vary across the prognostic groups. Interrogation of gene expression data from two clinical studies of ADT showed that ERβ mRNA expression increases following inhibition of AR signalling. This finding was confirmed *in vitro* by silencing AR expression in wild-type LNCaP prostate cancer cells.

The inducible LNCaP-ERβ cell line model was used to investigate the effect of sustaining ERβ signalling in early prostate cancer at phenotypic, transcriptomic and genomic levels. This revealed that when stimulated by a selective agonist (3β-adiol) in the presence of androgen-stimulated AR, ERβ decreases prostate cancer cell proliferation by widespread alteration of AR-dependent gene expression. Mechanistically, this reprogramming appears to occur by competition for key, highly transcriptionally active DNA-binding sites. A further possibility may be that ERβ sequesters nuclear receptor co-factors such as FOXA1 to modulate the activity of AR. These studies reveal for the first time two important findings that will inform future work in the field. Firstly, genome-wide ERβ bind-

ing in a prostate cancer context and secondly, genome-wide evidence of shared ERβ/AR binding sites detected in any cell- or tissue-context. Taken together, these findings suggest that activation of ERβ by a selective agonist in the early, androgen-sensitive stages of prostate cancer may slow or even abrogate disease progression.

Chapter 6

Discussion

6.1 The clinical rationale for studying estrogen biology in prostate cancer

Despite recent advances in all aspects of the clinical management of prostate cancer, ranging from improved imaging-guided diagnostics [Nelson et al., 2013], to the introduction of robotic-assisted radical prostatectomy [Abbou et al., 2001] and IMRT [Sheets et al., 2012] in primary treatment, men with prostate cancer remain at high risk of relapse following primary therapy [Paller and Antonarakis, 2013]. For those men that relapse and go on to develop CRPC, this disease remains incurable despite recent advances with the development of the AR-targeting therapies Enzalutamide and Abiraterone (reviewed in [Lamb et al., 2013]). There is therefore a clear need to further our understanding of the biology driving the development and progression of prostate cancer in order that the natural history of the disease can be modified early in its course to improve clinical outcomes.

Previous literature has established that estrogen, as well as androgen is essential for the development of prostate cancer and that estrogen exposure is a significant epidemiological risk factor for the disease (Section 1.3.1). A range of SERMs have already been developed and are in regular clinical use for the treatment of breast cancer. As these drugs are already approved for human use, they could be used to treat prostate cancer as long as the preclinical and clinical trial data are support-

ive. However, despite promising *in vitro* data showing a benefit to manipulating estrogen signalling in prostate cancer cell lines, there is no definitive clinical trial evidence proving that targeting estrogen receptor function can improve clinical outcomes (Sections 1.3.3 and 1.3.4). There is therefore, a disconnection between the *in vitro* and *in vivo* data, which requires explanation in order to further advance the field. The data presented in the current work suggests that both the use of inadequately validated antibodies and insufficiently characterised cell line models in previously published literature may have contributed to this position and actively hindered progress in furthering the understanding of ERβ biology in prostate cancer.

6.2 Validation of ERβ antibodies

Contradictions in expression profiles as determined by IHC and reported mechanisms of action and interactors as determined by antibody-based molecular biology methods have contributed to the uncertainty regarding the role of ERβ in prostate cancer. The lack of clear consensus regarding tissue expression of ERβ and correlation with clinico-pathological parameters such as Gleason grade is particularly problematic [Asgari and Morakabati, 2011; Attia and Ederveen, 2012; Bouchal et al., 2011; Dey et al., 2014; Horvath et al., 2001; Leav et al., 2001; Risbridger et al., 2007; Zellweger et al., 2013; Zhu et al., 2004].

The first aim of this study, therefore was to undertake a detailed assessment of ERβ antibodies using a multi-modal approach in a robust experimental model. This has demonstrated marked variation in the specificity of commonly used, commercially available ERβ antibodies for accurate detection of ERβ by Western blotting and the ChIP-based technique RIME. Arguably the most important finding of this work has been to show that NCL-ER-BETA, a very commonly used ERβ antibody was not specific to ERβ by either Western blotting or RIME (Figures 3.3 and 3.4). Much of the confusion in the ERβ field may be attributable to this antibody, which yields bands on a Western blot of appropriate size for ERβ, but we have now shown to be a non-specific interaction (Figure 4.7).

A further important finding has been to show clear experimental evidence of the fact that a particular antibody can be specific in one experimental approach but not another. The PPG5/10 antibody, which has been previously validated for IHC [Wu et al., 2012] and demonstrated detection of ERβ by RIME, but not Western blotting perfectly illustrates this. Our findings reassert the importance of validating antibodies for individual experimental assays [Baker, 2015; Bordeaux et al., 2010].

RIME was initially developed as a discovery tool to study the interacting proteomes of transcription factors in an unbiased manner [Mohammed et al., 2013]. The advantage of using RIME in antibody validation arises from being able to identify specific, named peptides purified by a particular antibody, rather than relying on the presence of a protein band of approximate size on a Western blot, or comparing tissue expression determined by IHC. Furthermore, RIME facilitates a head-to-head comparison of antibodies, to aid in the selection of which antibody to use in subsequent biological experiments. The identification of known ERβ interactors further validates the findings (Figure 3.5). HSP90 is a nuclear receptor chaperone protein known to interact with both ERα and ERβ [Powell et al., 2010]. Powell *et al.* showed (using the sc8974 antibody, which was specific to ERβ by Western blotting) that HSP90 is essential for the formation of ERα/α homodimers and ERα/β heterodimers. Ligand binding to the nuclear receptor results in a conformational change and dissociation from the HSP90 complex, leading to active transcription. The role of HSP90 with respect to the formation of ERβ/β homodimers however, is less clear. Powell *et al.* [Powell et al., 2010] suggest that whilst HSP90 is needed for estrogen-dependent ERβ/β transcriptional activity, perturbation of the HSP90 complex would be minimally disruptive to the formation of ERβ/β homodimers. Similarly, Schülke *et al.* showed that inhibiting HSP90 has little effect on ERβ activity, suggesting that ERα and ERβ may operate independently of HSP90 [Schülke et al., 2010]. DDX54 is a regulator of nuclear receptor transcriptional activity, and has been shown to be a co-repressor of ERβ transcription [Rajendran et al., 2003]. In the presence of estradiol, DDX54 was shown to repress the transcriptional activity of ERβ by

60-90% [Rajendran et al., 2003].

The antibody validation approach outlined in this work addresses the two previously mentioned, flawed assumptions made in previous antibody validation studies. Firstly, assessing an antibody by IHC relies upon *a priori* knowledge of the tissue-specific expression profile of the factor of interest, against which the staining of the antibody undergoing assessment can be compared. If however, as in the case of ERβ the tissue expression is not well-characterised or varies from one study to another, then any comparison made has the potential to be misleading. This also holds true for Western blotting-based approaches using cell lines 'known' to express ERβ, as demonstrated in section 4.2. An alternative to using RIME for antibody validation would be to use siRNA against the factor of interest in a cell line thought to express said factor, to provide positive and negative controls that can be tested by Western blotting and validated by RT-qPCR.

In conclusion, this antibody validation study facilitated an informed decision-making process with respect to selection of reliable antibodies for use in subsequent biological experiments. ERβ antibodies CWK-F12 and MC10 were previously validated by other approaches [Choi et al., 2001; Wu et al., 2012], and the results described here provide further confidence in their use for Western blotting, IP-based methods and IHC (for CWK-F12). Given that ERβ has been implicated as an important factor in breast, kidney, bladder, ovarian, colorectal, endometrial and non-small cell lung malignancies [Ciucci et al., 2014; Dey et al., 2013; Han et al., 2015; He et al., 2015; Hsu et al., 2013; Luo et al., 2015; Suzuki et al., 2008; Yu et al., 2013], the impact of this antibody validation work is likely to be wide-reaching.

6.3 Establishing an experimental model to study ERβ

6.3.1 Characterisation of existing prostate and breast cancer cell line models

This study has conclusively demonstrated that the low passage, genotyped prostate and breast cancer cell lines characterised do not express detectable levels of ERβ mRNA or protein. This multimodal approach has utilised four independent methods, two of which are antibody-dependent (Western blotting and RIME), and two of which are not (RT-qPCR and PRM) to provide thorough validation of the findings. It is particularly striking that LNCaP cells were not found to express ERβ, as this cell line model has been used extensively in previous studies of ERβ, with a wide and variable range of conclusions regarding its function drawn from the data. For example, Kim *et al.* showed that raloxifene, a selective estrogen-receptor modulator (SERM) induced apoptosis in LNCaP cells through activation of ERβ and induction of caspase-8 and -9 pathways [Kim et al., 2002b]. This same growth-inhibitory effect of raloxifene has been observed in other prostate cell lines thought to express ERβ (not included in the present study) [Rossi et al., 2011], suggesting it is reproducible. Others have shown that ERβ-mediated apoptosis in LNCaP cells may occur through the intrinsic pathway and upregulation of FOXO3a [Dey et al., 2014], reinforcing the view that ERβ has a predominantly growth-suppressive and pro-apoptotic role in prostate cancer. In direct contrast however, ERβ has been directly implicated as an oncogene and driver of non-androgenic, AR-dependent gene transcription in LNCaP cells [Yang et al., 2012]. In this study, the authors proposed that in castrate conditions, estrogen-stimulated ERβ can drive AR activity by indirect binding to AR with PELP1 acting as a linker protein. Whilst acknowledging the known variability across strains of immortalised cell lines [Masters, 2000], the work presented in this chapter suggests that previously published studies of the role of ERβ conducted in the most commonly used prostate cancer cell lines should be

interpreted with caution.

This study and others have suggested that tissue expression of ERβ is greatest in the basal epithelium of benign prostate glands (Figure 4.8A) [Horvath et al., 2001; Leav et al., 2001]. Expression then declines with the development of Gleason 3 prostate adenocarcinoma (Figure 4.8B), an observation that underpins the hypothesis that ERβ is predominantly tumour-suppressive. It is therefore logical that immortalised prostate cancer cell lines, which have been derived from metastatic tumour deposits originating from luminal epithelial cells will have lost ERβ expression as part of the transformation from benign to malignant phenotype. The moderate expression of ERβ observed in high grade prostate cancer (Figure 4.8D) may reflect increased expression of ERβ isoform 2, which has been reported in advanced and castrate-resistant disease [Dey et al., 2012; Leung et al., 2010]. However, our IHC data are not able to provide a definitive answer to this question as the CWK-F12 antibody recognises the N-terminal region common to all ERβ isoforms. Clearly, this is an important area for further investigation.

6.3.2 Development and validation of the LNCaP-ERβ cell line

In order to better understand the relationship between ERβ and AR and their respective roles in suppression or promotion of cancer progression it was therefore apparent that a new cell line model was required. In view of a number of studies implicating direct interplay and crosstalk between these receptors [Grubisha and DeFranco, 2013; Rizza et al., 2014; Yang et al., 2012] a system with inducible ERβ expression was derived to enable detailed study of the effects of each receptor upon the other. This cell line was developed in a lengthy, two-step process with introduction of the tetracycline-repressor, followed by clonal selection and assessment of the inducible 'switch' by *luciferase* assay. The clone with the lowest signal from the 'dox-off' condition was then selected for subsequent introduction of the ERβ plasmid. The multimodal characterisation of the resulting LNCaP-ERβ model comprehensively demonstrates expression of ERβ in the

'dox-on' condition and importantly, absence of ERβ expression in the 'dox-off' condition. This process ensured a clean model system, with no contamination of experimental controls by leaky ERβ expression. Morphologically the cells behave like parental LNCaP cells and have maintained expression of key factors such as FOXA1 and AR, which is important when considering the translational relevance of any data generated from this model system [Robinson et al., 2014].

6.3.3 Exploring the ERβ protein interactome in LNCaP-ERβ cells

The ERβ protein interactome is not well characterised, particularly in the context of prostate cancer. The RIME data presented permit a preliminary investigation into protein networks that may interact with ERβ in prostate cancer. Furthermore, the identification of 4 proteins known to interact with ERβ provides additional verification of the validity of the LNCaP-ERβ cell line model (Figure 4.1).

The known interaction between HSP90 and ERβ [Powell et al., 2010; Schülke et al., 2010] has previously been discussed (Section 6.2). TRIM24 (TIF1α) is a transcriptional co-activator, which is known to interact with a number of nuclear receptors, including ERβ [Thenot et al., 1999]. Thenot *et al.* (1999) showed that in the presence of estrogen TRIM24 was shown to interact with ERα bound to ERE, whereas in the absence of hormone, TRIM24 interacted preferentially with ERβ independently of the ERE, suggesting differing roles for the co-factor and two receptors under differing hormonal contexts. NRIP1 (RIP140) is a co-regulator of transcriptional activation by nuclear receptors such as glucocorticoid receptor (GR) [Windahl et al., 1999], mineralocorticoid receptor (MR) [Hellal-Levy et al., 2000] and ERα [Cavailles et al., 1995]. In MCF-7 cells stably expressing ERβ, NRIP1 has been shown to be part of a network of proteins interacting with ERβ, which also includes PELP1 [Nassa et al., 2011]. Furthermore, ERβ and NRIP1 have been shown to share approximately 9000 DNA binding sites in MCF-7-ERβ cells, with many of the shared sites relating to apoptosis, growth suppression and

metabolic factors such as fatty acid metabolism, inflammation and regulation of NFκB for example. Interestingly, the authors describe an ERβ/NRIP1-related gene signature, which is predictive of better outcome in breast cancer [Madak-Erdogan et al., 2013]. HMGB1 is a chromatin regulatory factor, which has been shown to be downregulated by ligand-activated ERβ [Dey et al., 2012]. Down-regulation of HMGB1 has been shown to contribute towards apoptosis in LNCaP cells [Tang et al., 2010] and it is known to interact with the other nuclear steroid hormone receptors AR, PR and GR [Boonyaratanakornkit et al., 1998].

A number of proteins identified in the RIME data are not currently known to interact with ERβ, but are known to interact with other nuclear steroid hormone receptors. For example, FKBP5 is part of the HSP90 chaperone complex responsible for trafficking AR [Schülke et al., 2010] and ERα [Nair et al., 1996] from the cytoplasm into the cell nucleus upon ligand activation. Another co-chaperone protein, FKBP4 is known to participate in the HSP90 complex and interact with GR, ERα, MR, PR and AR [Nair et al., 1996; Schülke et al., 2010; Taipale et al., 2014]. HOXB13 is involved in embryological development of the prostate [Eeles et al., 2013] and has been shown recently to interact with AR in the earliest stages of malignant transformation, acting with FOXA1 to reprogram the AR cistrome to a cancer-associated phenotype [Pomerantz et al., 2015]. Furthermore, a rare variant of HOXB13 has been shown to significantly increase prostate cancer risk and has been found in families with multiple cases of prostate cancer [Eeles et al., 2013].

Signal transducer and activator of transcription (STAT) 3 is an important transcriptional activator that mediates cells' responses to interleukins and other growth factors. It is known to interact with nuclear receptors GR [Zhang et al., 1997], ERα [Wang et al., 2001], PR [Proietti et al., 2011] and AR [Matsuda et al., 2001]. Inhibition of STAT3 in a prostate cell line (DU145) xenograft model reduced tumour growth, cell viability and invasion by induction of caspase-dependent and -independent apoptosis [Canesin et al., 2016]. STAT3 is also known to interact with a number of established ERβ interactors; namely TWIST1 [Grossmann et al., 2015] and RUNX2 [Fan et al., 2007]. These markers of EMT are thought

to be downregulated by ERβ. As ERβ expression falls with the development of cancer, expression of TWIST1 and RUNX1 increase leading to activation of the EMT program and increased propensity to metastatic progression [Dey et al., 2012].

These data provide the opportunity to begin building up a putative network of ERβ interacting proteins. Further work would be required to prove definitive physical interactions between these proteins and understand the functional consequences and interrelationships of the component proteins within the network. The LNCaP-ERβ cell line model developed in this work has been shown to be a valid *in vitro* preclinical model system for testing the overall study hypothesis that ERβ modulates processes involved in prostate carcinogenesis.

6.4 Genomic crosstalk between ERβ and AR

As discussed throughout this thesis, there has been confusion in the published literature regarding the predominant role of ERβ in prostate, and other cancer types. The present work clearly demonstrates that much of this confusion has arisen as a consequence of using poorly validated reagents and inadequately characterised cell line models. Having developed a 'toolbox' of validated reagents and a fully characterised cell line model, the original study hypothesis could then be tested in a robust manner, with the principle aim of further understanding the relationship between ERβ and AR in prostate cancer development.

6.4.1 Findings from clinical data

The clinical rationale for investigating ERβ was established afresh by demonstrating variability in ERβ expression across prognostic groups generated from clinical material [Ross-Adams et al., 2015], which clearly showed that greater ERβ expression is associated with improved prognosis (in terms of BCR-free survival). This finding is in keeping with most of the previous literature, which suggests that ERβ is a tumour-suppressor in prostate cancer. Secondly, by show-

ing an increase in ERβ expression following silencing of AR activity by ADT, [Rajan et al., 2014; Shaw et al., 2016] we gain initial insight into the relationship between ERβ and AR. These data indicate that AR represses ERβ expression, suggesting that as AR expression increases during malignant transformation of the prostate, there is a resulting decrease in ERβ expression and subsequent loss of its tumour-suppressive effect. This would explain the previously observed pattern of greater ERβ expression in benign *versus* malignant tissues [Asgari and Morakabati, 2011; Bonkhoff et al., 1999; Dey et al., 2014; Horvath et al., 2001; Leav et al., 2001].

6.4.2 Revealing the genomic activity of ERβ in prostate cancer

There is however, a paucity of data on the detailed mechanisms by which ERβ acts in the prostate, particularly at a genomic level. In the present study, genome-wide DNA binding of ERβ in a prostate cancer context has been successfully demonstrated for the first time. Furthermore, to our knowledge this is the first genome-wide study in any context to show that ERβ and AR compete for DNA-binding sites, resulting in widespread changes in gene expression and inhibition of cell proliferation (Figure 6.1).

In the nuclear receptor field, it is becoming increasingly apparent that crosstalk between different receptors is a biologically and clinically important phenomenon that needs to be accounted for in future studies and clinical trials [Arora et al., 2013; Mohammed et al., 2015; Rizza et al., 2014]. From the data presented here, it was very interesting to note that ERβ only exerts its effects on proliferation, gene expression and DNA-binding when it is ligand-bound *in the presence of* androgen-activated AR. This mirrors exactly previously published work regarding the effect of PR on ERα-dependent breast cancer, whereby PR is anti-proliferative when bound by its natural ligand in the presence of estrogen-stimulated ERα [Mohammed et al., 2015]. It is, however important to ensure that the effects of dual-hormone stimulation observed in this work are not the consequence of overloading

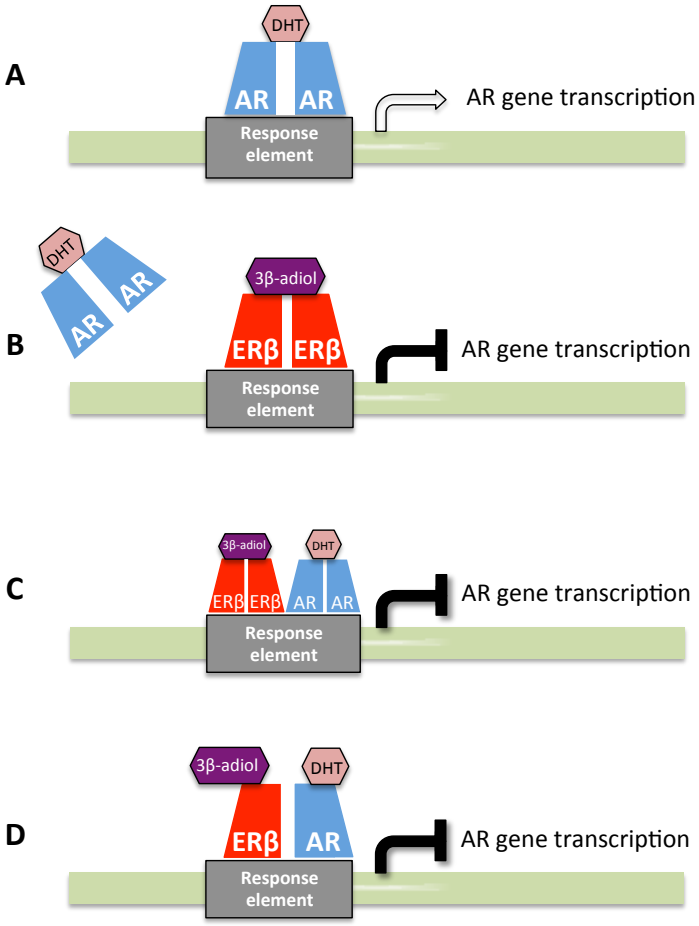


Figure 6.1: **Possible models of competitive antagonism between ERβ and AR.** (A) Under androgen-stimulated conditions, AR dimerises and binds to DNA response elements to activate AR-dependent transcription. The presence of ligand-activated ERβ is likely to perturb this mechanism by one of two ways. Either ERβ homodimers displace AR from the chromatin (B) or ERβ and AR co-bind at shared binding sites (C). A further, although less probable mechanism is that ERβ/AR heterodimers form and bind to shared sites, with resulting inhibition of AR-dependent transcription (D). Further study is required to determine these mechanisms in detail. AR, androgen receptor; DHT, dihydrotestosterone; ERβ, estrogen receptor β.

the model system with active nuclear receptors, such that pro-proliferative doses of ligands become anti-proliferative or *vice versa*; a phenomenon that has been

previously described in prostate cancer xenograft models [Chen et al., 2004]. Repeating the LNCaP-ERβ cell line studies presented in Chapter 5 with reducing doses of R1881 and 3β-adiol would address this concern.

Our findings support the view that ERβ is tumour-suppressive in prostate cancer and that targeting it with an agonist may be a viable way of modifying progression of the disease early in its natural history. The data presented in this work suggest that ERβ and AR reciprocally inhibit one another. The exact mechanistic details of this remain unclear, however competition for shared DNA-binding sites and key co-transcription factors such as FOXA1 are likely to be important factors. Further studies are required to test and validate these findings.

Two recently published studies [Gehrig et al., 2017; Wu et al., 2017] confirm a number of our findings. Wu *et al.* (2017) showed that agonist-stimulated ERβ down-regulates AR signalling through increased expression of an AR co-repressor complex (DACH1/2), and upregulates expression of the tumour suppressor PTEN. Secondly, Gehrig *et al.* (2017) showed that AR represses ERβ expression, and activation of ERβ with a selective agonist decreases AR expression leading to decreased cell survival. Interestingly, these findings were generated from androgen-resistant cell lines representative of CRPC, which is in contrast to our own data which suggest that targeting ERβ is only effective in androgen-replete conditions. Clearly, further study is required, as understanding the details of this biology will have implications for how and and when an ERβ agonist could be used clinically.

6.5 Future directions

Experimental validation

With the assistance of collaborators Dr. Luke Selth and Prof. Wayne Tilley (University of Adelaide, Australia) a number of further experiments are planned to further test the study hypothesis and validate the data presented in this work.

1. **AR ChIP-seq in LNCaP-ERβ cells.** As discussed in section 5.2.7, our original intention was to perform both ERβ and AR ChIP-seq in the LNCaP-ERβ cell line under androgen-, estrogen- and dual-hormone-treated conditions in order to demonstrate reciprocal changes in the DNA-binding patterns of each factor as influenced by the other. Due to the scale of this experiment, it was not possible to repeat this within the available timeframe after failure of the AR antibody. This is a key experiment, which should reveal further mechanistic insight into the relationship between ERβ and AR at a genomic level.

2. **ERβ and AR RIME in LNCaP-ERβ cells.** In order to establish whether ERβ and AR compete for key co-factors such as FOXA1 under the various hormone treatments, quantitative RIME under matched experimental conditions to the RNA- and ChIP-seq experiments will reveal and quantify the protein interactome of each nuclear receptor. This will establish whether ligand activation of each receptor sequesters available co-factors to alter the genomic activity of the other.

3. **Treatment of *ex vivo* prostate tumours with an ERβ agonist, with or without AR inhibition.** The aim of this experiment is to validate the findings generated from the LNCaP-ERβ cell line in an *in vivo* context and provide data from a more clinically-relevant experimental model. The results of this experiment would provide a key link from the *in vitro* to an *in vivo* context, and potentially provide the basis for a clinical trial of an ERβ-specific agonist in prostate cancer.

Therapeutic output

If these validations are successful in confirming the findings presented in this thesis, then there will be a clear rationale for a clinical trial of an ERβ agonist in men with prostate cancer. These data suggest that activated ERβ inhibits cell proliferation and alters AR-dependent gene expression in the presence of androgen-activated AR. It would therefore seem logical that an ERβ agonist would be most useful in the early, androgen-sensitive stage of prostate cancer. An ERβ agonist

could foreseeably be administered to men with intermediate risk disease on active surveillance (Section 1.1), with the intention that this would slow or prevent further progression of the disease by modulating the activity of oncogenic AR in an indirect manner to alter the natural history of the disease. Alternatively, men with high risk or locally advanced disease who are commenced on ADT could receive an ERβ agonist concurrently to further suppress the disease and reduce the inevitable selection pressure towards CRPC exerted by conventional ADT alone [Claessens et al., 2014; Scher et al., 2004]. An ERβ agonist may have an additional anti-inflammatory effect, which could also contribute towards reducing disease progression (Section 1.5.1) [Warner et al., 2017].

6.6 Conclusions

The work presented in this thesis has demonstrated that much of the confusion in the existing literature regarding the role of ERβ is likely to be the result of utilisation of inadequately validated antibodies. Using a robust cell line model with inducible ERβ expression, two ERβ antibodies have been validated by multiple experimental methods for use in Western blotting, immunoprecipitation and IHC.

Using these validated antibodies and additional antibody-indepent methods, multiple cell line models previously used to study ERβ have been shown to lack its expression. This casts doubt on many studies previously published on ERβ biology. An androgen-sensitive prostate cancer cell line with inducible ERβ expression was developed and validated by multiple experimental techniques. A putative network of ERβ-interacting proteins has been revealed, providing opportunities for further study and validation of the findings in a prostate cancer context. This cell line model will be a vital resource for ongoing studies of the role of ERβ in prostate cancer.

In clinical samples, ERβ appears to be down-regulated by AR. This may result from increased expression of AR known to occur in malignant transformation

of prostate epithelium. In cases where ERβ expression is maintained, there is an associated improvement in BCR-free survival. *In vitro* studies demonstrated that ligand-activated ERβ decreases cell proliferation and down-regulates AR-dependent gene expression through competition for transcriptionally active binding sites shared by ERβ and AR. These studies have demonstrated both genome-wide ERβ binding sites in a prostate cancer context and ERβ/AR cross-talk mediated by competition for shared binding sites for the first time. The data presented in this thesis suggest that in future, it may be possible to administer an ERβ agonist to men with prostate cancer to slow or abrogate progression of their disease.

Appendix A

Publications authored during PhD:

1. **A.W. Nelson**, W.D. Tilley, D.E. Neal, J.S. Carroll. Estrogen receptor beta in prostate cancer: Friend or Foe? *Endocr Relat Cancer.* 2014;21(4):T219-34

2. **A.W. Nelson**, A.J. Groen, J.L. Miller, A.Y. Warren, K.A. Holmes, G.A. Tarulli, W.D. Tilley, B.S. Katzenellenbogen, J.R. Hawse, V.J. Gnanapragasam, J.S. Carroll. Comprehensive assessment of estrogen receptor beta antibodies in cancer cell line models and tissue reveals critical limitations in reagent specificity. *Mol Cell Endocrinol.* 2017;440 138-150

Estrogen receptor beta in prostate cancer: friend or foe?

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Abstract

Prostate cancer is the commonest, non-cutaneous cancer in men. At present, there is no cure for the advanced, castration-resistant form of the disease. Estrogen has been shown to be important in prostate carcinogenesis, with evidence resulting from epidemiological, cancer cell line, human tissue and animal studies. The prostate expresses both estrogen receptor alpha (ERA) and estrogen receptor beta (ERB). Most evidence suggests that ERA mediates the harmful effects of estrogen in the prostate, whereas ERB is tumour suppressive, but trials of ERB-selective agents have not translated into improved clinical outcomes. The role of ERB in the prostate remains unclear and there is increasing evidence that isoforms of ERB may be oncogenic. Detailed study of ERB and ERB isoforms in the prostate is required to establish their cell-specific roles, in order to determine if therapies can be directed towards ERB-dependent pathways. In this review, we summarise evidence on the role of ERB in prostate cancer and highlight areas for future research.

Key Words
- estrogen receptor beta
- prostate
- cancer
- androgen
- estrogen

Endocrine-Related Cancer (2014) **21**, T1–T16

Introduction

Prostate cancer is the commonest, non-cutaneous cancer in men, affecting 214 per 1000 European men. It is the second commonest cause of cancer death, accounting for 15% of all male cancers in developed countries (Heidenreich *et al.* 2011, Mottet *et al.* 2011). Ever since the landmark research of Huggins and Hodges (Huggins 1943, Huggins & Hodges 1972) demonstrating the importance of steroid hormones in the development and treatment of prostate cancer, there has been interest in the effects of estrogen on the prostate gland. Initially, hormone treatment for prostate cancer involved manipulation of systemic hormone levels with exogenous estrogen therapy (in the form of high-dose diethylstilbestrol) to suppress androgen production indirectly via the hypothalamo–pituitary–gonadal axis (Huggins & Hodges 1972). However, the unacceptably high rates of cardiovascular side effects associated with systemic estrogen therapy, coupled with the advent of alternative treatment options, resulted in reduced use of this therapy (Morales & Pujari 1975). Consequently, for a period of time, interest in understanding the effects of estrogen in the prostate also declined. Hormonal suppression of prostate cancer is now primarily achieved with androgen deprivation therapy (ADT) involving synthetic leutinising-hormone-releasing-hormone (LHRH) analogues, which suppress androgen production via negative feedback inhibition of the hypothalamic–pituitary–gonadal axis (Heidenreich *et al.* 2011). Although most prostate tumours initially respond well to ADT, after a period of time prostate cancer inevitably ceases to respond to androgen deprivation. Disease progression with ADT is

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termed castration-resistant prostate cancer (CRPC) (Scher *et al*. 2004), and is characterised by altered androgen receptor (AR) signalling. Possible mechanisms for this include amplification (Bubendorf *et al*. 1999) or mutation of the AR gene, stabilisation of AR protein (Holzbeierlein *et al*. 2004), altered expression of AR-coregulators (Chmelar *et al*. 2007), generation of constitutively active AR splice variants (Dehm *et al*. 2008, Hu *et al*. 2012) and increased intratumoural androgen biosynthesis (Cai & Balk 2011, Ishizaki *et al*. 2013), all of which contribute to the maintenance of AR-dependent transcription in a castrate environment (Knudsen & Penning 2010). Genome-wide mapping of AR DNA-binding has shown that in CRPC AR binds to new sites on the DNA, resulting in an alternative transcriptional programme to that seen in primary disease (Sharma *et al*. 2013). CRPC carries a poor prognosis and a median survival of 18 months from diagnosis (Wu *et al*. 2007). New generation chemotherapeutics agents targeting AR signalling such as abiraterone and enzalutamide have led to modest improvement in prostate cancer survival, but they are not curative (Attard *et al*. 2011, Lamb *et al*. 2013).

In recent years, with advances in the understanding of AR function in CRPC and the cross-talk that occurs between AR and estrogen receptor alpha (ERA) in prostate cancer (Grubisha & Defranco 2013) and specific subtypes of breast cancer (Robinson *et al*. 2011), there has been renewed interest in understanding ER biology in the prostate and its role in prostate cancer. Further interest has arisen as a result of recent phase 2 clinic trial evidence supporting the use of transdermal estrogen therapy in prostate cancer, which avoids first-pass liver metabolism and has an improved side-effect profile over both parenteral estrogens and LHRH analogues (Langley *et al*. 2013). It is now known that the prostate gland expresses both ERA and estrogen receptor beta (ERB) (Horvath *et al*. 2001, Celhay *et al*. 2010). ERB is expressed in a wide range of reproductive and non-reproductive tissues including the CNS, cardiovascular system, gastrointestinal tract, urogenital tract (male and female) and skeleton (Bottner *et al*. 2014). The physiological role of ERB in each of these tissues has not been fully elucidated, but it has been implicated in the regulation of glucose homeostasis and insulin signalling and may also modulate immunologically mediated inflammatory pathways (Harris *et al*. 2003, Foryst-Ludwig *et al*. 2008). In addition, ERB is considered a negative regulator of ERA, acting to modulate transcriptional responses to estrogen in a tissue- and cell-context dependent manner (Bottner *et al*. 2014).

The traditional paradigm regarding the roles of the two ERs in the prostate is that ERB is predominantly protective, being anti-carcinogenic and pro-apoptotic (Chang & Prins 1999, Horvath *et al*. 2001, Zhu *et al*. 2004, Ellem & Risbridger 2007, McPherson *et al*. 2010, Muthusamy *et al*. 2011, Nakajima *et al*. 2011, Attia & Ederveen 2012), whereas ERA is oncogenic and promotes cell proliferation and survival (Ellem & Risbridger 2007, Risbridger *et al*. 2007, McPherson *et al*. 2008, Bonkhoff & Berges 2009, Celhay *et al*. 2010, Attia & Ederveen 2012). This view is based on a range of observations including epidemiological and *in vivo* studies, preclinical drug trials and expression profiles of the two ERs in human prostate cancer. However, much of the published data regarding the role and function of ERB appear to be conflicting, with studies conducted in cancer cell lines (McPherson *et al*. 2010, Dey *et al*. 2012, Yang *et al*. 2012), rodent models (Ricke *et al*. 2008, Attia & Ederveen 2012) or human tissues (Horvath *et al*. 2001, Celhay *et al*. 2010, Leung *et al*. 2010, Hussain *et al*. 2012) generating apparently contradictory results. Thus the precise actions of ERB in the prostate remain to be completely elucidated (Shaaban *et al*. 2003, Risbridger *et al*. 2007, Zhao *et al*. 2007, Celhay *et al*. 2010, Nelles *et al*. 2011, Dey *et al*. 2012, Yang *et al*. 2012). In this article, we review recent research in the area of ERB biology, with particular attention to its relevance in clinical aspects of human prostate cancer, and highlight areas for future research.

Evidence for the role of estrogen in prostate cancer: epidemiological

It is well established that European men have a lower risk of developing prostate cancer than African-American men, and that for Japanese men the lifetime risk of developing prostate cancer is lower still (de Jong *et al*. 1991, Ross *et al*. 1992, Ellem & Risbridger 2007). Two interesting observations are noteworthy in this respect. First, there are no significant differences in levels of circulating testosterone between these three ethnic groups (Ross *et al*. 1992), whereas levels of serum estrogens are higher in black men as compared with Caucasian men (Rohrmann *et al*. 2007, Abd Elmageed *et al*. 2013). However, a direct correlation between serum estrogen levels and prostate cancer risk has not been demonstrated (Yao *et al*. 2011, Bosland 2013). Secondly, the age of peak prostate cancer incidence occurs at a time when serum testosterone levels are in decline, but estrogen levels remain constant (Vermeulen *et al*. 2002). This has lead to the hypothesis that it is the ratio of serum estrogen to

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testosterone, rather than the absolute values of each, which gives rise to the observed differences in prostate cancer risk between the various ethnic groups (Ellem & Risbridger 2007, Bosland 2013). However, recent technological advances, which now permit more accurate measurement of both ultra-low and intra-tissue hormone levels, mean that a contemporary reassessment of this subject is needed (Barth *et al.* 2010, Hickey & Norman 2010, Stener-Victorin *et al.* 2010). Determining the actual levels of estrogen and androgen metabolites in the prostate will be essential for understanding ERA, ERB and AR behaviour. Racial differences in levels of prostatic ERB expression as measured by immunohistochemistry have been demonstrated by Abd Elmageed *et al.* (2013) who showed that the frequency of ERB immunostaining in prostate tumours was significantly higher in black men than in Caucasian men, which in turn, correlated with poorer clinical outcome.

Estrogen-related prostate cancer risk has been linked to dietary factors (Hori *et al.* 2011). The traditional Japanese diet contains high levels of dietary phytoestrogens, which have been shown in prostate cancer cell lines to upregulate ERB activity resulting in decreased expression of AR (Thelen *et al.* 2005, 2007, Stettner *et al.* 2007) and induction of G1-cell cycle block (Shen *et al.* 2000). In rat models, phytoestrogens can induce prostate epithelial cell apoptosis (Attia & Ederveen 2012), thereby demonstrating protective effects against prostate cancer. Phytoestrogens, along with other dietary estrogens such as lignans, flavonoids and lipoflavinoid are known to have up to 30-fold greater affinity for ERB than ERA, and are thought to promote the beneficial, protective effects of estrogen in the prostate (Kuiper *et al.* 1998, Ellem & Risbridger 2007, Thelen *et al.* 2014). A large population-based case–control study from Sweden demonstrated a reduced incidence of prostate cancer in those with a diet rich in phytoestrogens (Hedelin *et al.* 2006). Incidence of prostate cancer in Japan has been rising since the 1940s, coinciding with increased 'westernisation' of the Japanese diet. Specifically, it has been proposed that the 20-fold increase in the consumption of milk and animal fat, both of which contain estrogens with a high affinity for ERA (mediating the adverse effects of estrogen) may explain some of the recent rise in prostate cancer incidence among Japanese men (Ganmaa *et al.* 2003, Carruba 2007). There are, however, conflicting results in the literature regarding the effects of phytoestrogens in prostate cancer, as genistein (a highly ERB-specific isoflavone (Jiang *et al.* 2013)) has been shown in a study of prostate cancer xenograft-bearing mice to promote the development of metastatic disease progression in an ERB-dependent manner (Nakamura *et al.* 2013) (discussed further in section 'Evidence for the role of estrogen in prostate cancer: drug trials').

Evidence for the role of estrogen in prostate cancer: animal studies

Some of the most compelling evidence for the importance of estrogen in prostate carcinogenesis comes from a series of animal studies (Ricke *et al.* 2008) (Fig. 1). Ricke *et al.* demonstrated in mice that androgen, estrogen, aromatase and ERA are all required for prostate carcinogenesis. Aromatase is a highly substrate-specific cytochrome p450 enzyme, found in the membrane of the endoplasmic reticulum, which has the unique function in vertebrates of being able to convert androgens to estrogens (Ghosh *et al.* 2009). In normal prostate, aromatase is expressed in the stromal cells and is responsible for local paracrine conversion of androgen to estrogen (Risbridger *et al.* 2007).

The necessity for estrogen in prostate carcinogenesis was demonstrated in experiments where either androgen alone, or androgen and estrogen were administered to aromatase knockout (ArKO) mice (Ricke *et al.* 2008). ArKO mice given androgen and estrogen developed prostatic intraepithelial neoplasia (PIN – a premalignant histological phenotype which, especially when high grade or multifocal, is a recognised risk factor for the development of invasive prostate cancer (Nelson *et al.* 2003, Merrimen *et al.* 2009)), whereas ArKO mice given androgen alone had no such change (Ricke *et al.* 2008). From this, the authors concluded that local production of estrogen within the prostate, facilitated by aromatase-mediated conversion of androgen to estrogen, was likely to be

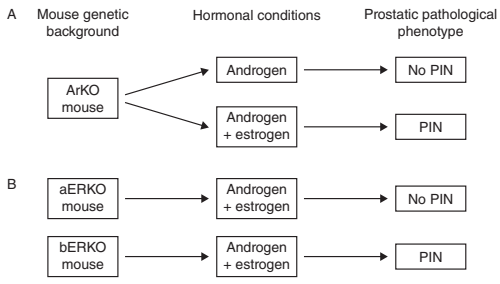

Figure 1
Summary of animal studies conducted by Ricke *et al.* (2008), demonstrating: (A) in order for prostate intraepithelial neoplasia (PIN) to arise it is necessary for androgen, estrogen and functional aromatase all to be present; (B) this is an ERA-mediated process, which is suppressed by ERB. ArKO, aromatase knockout; aERKO, ERA knockout; bERKO, ERB knockout.

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a significant factor in prostate carcinogenesis. Epithelial expression of aromatase is upregulated in prostate cancer (Ellem *et al.* 2004, Celhay *et al.* 2010), a process that is driven by gene promoters (I.3, I.4 and PII) responsive to inflammatory cytokines (Santen *et al.* 1997, Zhao *et al.* 1997, Shozu *et al.* 2000). The implication of this is twofold; first, increased local production of estrogens with resulting carcinogenesis and, secondly, the establishment of a 'positive feedback loop' between aromatase, estrogen and tissue inflammation (Ellem & Risbridger 2007). Indeed, high expression levels of aromatase and aromatase gene polymorphisms in early onset primary human prostate cancer have been found to correlate with decreased time to disease relapse, further underlining its importance in prostate carcinogenesis (Cussenot *et al.* 2007, Celhay *et al.* 2010).

In order to determine which of the ERs is responsible for mediating adverse effects of estrogen, Ricke *et al.* (2008) administered testosterone and estrogen to ERB knockout (bERKO) or ERA knockout (aERKO) mice. There was no difference between wild type (WT) and bERKO mice receiving hormone treatment, whereas aERKO mice did not develop PIN, suggesting that ERA mediated this particular adverse effect of estrogen in the prostate (Ricke *et al.* 2008). Similar studies in intact rats demonstrated that testosterone alone is insufficient for the development of PIN; it was only with the addition of the selective ERA agonist, ERA-45, that PIN developed (ERA-45 is reported to have a 286-fold greater affinity for ERA than ERB (Attia & Ederveen 2012)). However, with the administration of testosterone, ERA-45 and an ERB-selective agonist (ERB-26), the onset of PIN was prevented (Attia & Ederveen 2012), demonstrating the differential function of the two ERs in prostate carcinogenesis. These findings must be interpreted with some caution due to the ongoing debate in the field regarding the phenotypic variability and validity of the bERKO mouse models used in previous studies (Harris 2007).

Evidence for the role of estrogen in prostate cancer: drug trials

ERs are attractive targets for prostate cancer treatment as therapeutic agents are already in existence and are widely used in hormone-dependent breast cancer (Lumachi *et al.* 2011). Raloxifene, a selective ER modulator (SERM), has been shown to induce cellular apoptosis and nuclear fragmentation in both androgen-sensitive and androgen-independent prostate cancer cell lines through activation of ERB, suppression of ERA and subsequent induction of the caspase-8 and -9 pathways (Kim *et al.* 2002*a*,*b*, Rossi *et al.* 2011). Studies of selective ERB agonists on prostate cancer cell lines have also been encouraging. Several investigators have demonstrated that selective ERB agonists will induce cystic atrophy in basal cells of the prostatic epithelium (McPherson *et al.* 2010, Hussain *et al.* 2012). These basal cells do not express AR (Ruizeveld de Winter *et al.* 1991), and therefore, are unaffected by conventional ADT. Thus, once ADT is withdrawn, the prostatic epithelium is able to regenerate from this basal cell population. Administration of an ERB agonist, however, perturbs this regenerative process resulting in cellular apoptosis via the extrinsic pathway, mediated by tumour necrosis factor α (TNFA). This, importantly, is an androgen-independent process and may therefore be relevant to the treatment of CRPC (McPherson *et al.* 2010, Hussain *et al.* 2012). Recent research has shown that ERB-mediated cellular apoptosis may also occur through the intrinsic pathway, via upregulation of the Forkhead protein, FOXO3a, which itself is regulated by PTEN. Deletion mutations of PTEN result in inhibition of the apoptotic mechanism of FoxO3a, providing a possible ERB-mediated mechanism by which PTEN mutations in prostate cancer contribute to cancer growth (Dey *et al.* 2013*b*).

In vitro studies of the effects of dietary phytoestrogens on prostate cancer cell lines have shown conflicting results. On the one hand it has been shown that administration of ERB-selective phytoestrogens in CRPC will revert cancer cells to a less malignant phenotype (Wuttke *et al.* 2002, Messina 2010, Andres *et al.* 2011, Reiter *et al.* 2011). This may be due to a number of mechanisms. It is known that mutated forms of AR are upregulated in CRPC allowing AR to continue driving prostate cancer growth and progression in the absence of androgen (Scher *et al.* 2004, Waltering *et al.* 2012). Upregulation of ERB in response to phytoestrogens results in down-regulation of AR, with a subsequent decline in serum levels of prostate-specific antigen (PSA) and other AR-dependent genes (Thelen *et al.* 2005, 2007). ERB may therefore function as a negative regulator of AR, as well as ERA. However, as previously mentioned, in a study conducted using a patient-derived prostate cancer tissue line mouse xenograft model (maintained by serial transplantation of sub-renal capsule xenografts) (Andersen *et al.* 2010), the ERB-selective phytoestrogen genistein has been shown to promote development of metastatic disease (Nakamura *et al.* 2013). The explanation for these conflicting results is not presently clear; however it has been hypothesised that in the mouse xenograft tumours, increased expression of metallothionein proteins in response to genistein-induced ERB activity

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may contribute to tumour invasion and metastasis (Nakamura *et al.* 2013); an interaction, which may not be reflected in cell-line studies where the tumour microenvironment is absent.

A promising clinical study was published by Price *et al.* (2006). In a phase 2b clinical trial of 514 men with biopsy-proven high-grade PIN, toremifene, an ERA-selective antagonist (Kangas 1990), was shown to reduce the incidence of invasive prostate cancer at 12 months by 48% vs placebo, thus preventing 6.8% cancers per 100 men per year. However, the outcome of this trial was reported after only a 12-month study period and no long-term data on the use of toremifene in prostate cancer have since been generated to address potential long-term side effects or duration of treatment benefit.

Trials of other ER modulators have also been unsuccessful. Fulvestrant, an ERA antagonist, has been shown to be effective in preclinical models with growth inhibition of prostate cancer cell lines (Lau *et al.* 2000, Leung *et al.* 2006*a*). However, in a phase 2 study of 20 men with CRPC, fulvestrant failed to produce either a clinical or biochemical (PSA) response (Chadha *et al.* 2008). Similarly, tamoxifen, a mixed ERA agonist/antagonist has been shown to be ineffective in men with CRPC (Bergan *et al.* 1999) despite inhibiting the growth of prostate cancer cell lines in preclinical studies (Rohlff *et al.* 1998). The reasons underlying these observations are not presently clear; however, it is likely that prostate cancer cell lines used in the preclinical studies are not reflecting the complex cross-talk between AR, ERA and ERB, and other stromal–epithelial interactions known to occur *in vivo* (Hanahan & Weinberg 2011, Robinson *et al.* 2011, Grubisha & Defranco 2013, Madak-Erdogan *et al.* 2013). These studies highlight the critical need for improved preclinical models of prostate cancer, in which to test new therapeutic agents targeted to the ERs.

ERs in the prostate

ERB was first identified by Kuiper *et al.* (1996) in the rat prostate. In humans, it is a 55 kDa protein encoded by the *ESR2* gene located on chromosome 14 (Enmark *et al.* 1997). Expression of ERB is regulated epigenetically by a CpG island in the promotor region (Zhu *et al.* 2004) and ERB expression is silenced by DNA-hypermethylation of the promotor (Zhao *et al.* 2003, Rody *et al.* 2005). ERB is strongly expressed in the basal and secretory compartments of benign prostate epithelium in both rodents and humans (Horvath *et al.* 2001). The principle ligand of ERB in the prostate is 5α-androstane-3β,17β-diol (3β-diol), a metabolite of 5α-dihydrotestosterone (DHT) (Oliveira *et al.* 2007). In prostate cell lines (benign and cancer) ERB has been shown to maintain differentiation of epithelial cells by regulation of epithelial–mesenchymal transition (EMT) genes such as Twist via hypoxia-inducible factor 1 alpha (HIF-1A) (Mak *et al.* 2013).

The gene coding human ERA (*ESR1*) is located on chromosome 6 (Menasce *et al.* 1993). In the prostate (rodent and human) ERA is predominantly expressed in the stroma (Celhay *et al.* 2010, Attia & Ederveen 2012). *In utero* studies of prostate development in rodents have shown that ERA expression appears before ERB, and excessive estrogenisation of the developing prostate (mediated via ERA) results in permanent changes in the prostate including squamous metaplasia, inflammation and epithelial dysplasia (Arai *et al.* 1978, Prins & Birch 1997). This 'imprinting' results in increased risk of a premalignant phenotype and prostate carcinogenesis (Prins *et al.* 2006, 2007, McPherson *et al.* 2008, Prins & Korach 2008). Although expressed from different genes, ERA and ERB share substantial sequence homology, in particular the DNA-binding domains (DBD) of the two receptors are 97% identical. This allows both ERs to recognise a consensus estrogen response element (ERE) on DNA with equal affinity (Le *et al.* 2013).

The advent of genome-wide transcription factor mapping by chromatin immunoprecipitation coupled with high-throughput sequencing (ChIP-seq) has enabled detailed study of how transcription factors such as steroid hormone receptors function, by revealing the locations of their DNA-binding sites (Carroll *et al.* 2006). We now know, for example, that in addition to proximal gene promotors, ERA and AR bind to distal enhancer elements, far from gene-transcription start sites, and by recruitment of co-regulatory factors initiate gene transcription by long-range chromatin interactions (Carroll *et al.* 2005, Wang *et al.* 2007, Massie *et al.* 2011). For ERA and AR, a number of these co-regulatory factors are now well-characterised and represent potential therapeutic targets (Carroll *et al.* 2005, Wang *et al.* 2007, 2009, 2011, Hurtado *et al.* 2011, Robinson *et al.* 2011, Sahu *et al.* 2011). Genome-wide mapping of both tagged (Zhao *et al.* 2010) and force-expressed recombinant ERB (Madak-Erdogan *et al.* 2013) DNA-binding in the MCF7 breast cancer cell line has demonstrated significant overlap between ERA and ERB DNA-binding sites, inferring complex cross-talk between the two receptors. In addition, there is evidence that ERB binds to distal enhancer elements in the same manner as ERA and AR to regulate gene expression (Carroll *et al.* 2005, Zhao *et al.* 2010, Massie *et al.* 2011). Despite these

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significant insights, there is still very limited understanding of the mechanisms and co-regulators by which ERB activity may be modulated and thus the resulting effects on ERB transcription.

ER expression in prostate cancer

In normal prostate, ERA expression is confined to the prostatic stroma (Tilley *et al.* 1985, Wernert *et al.* 1988, Leav *et al.* 2001). In contrast to ERB, ERA mRNA has been detected in high-grade PIN of the prostate, and ERA expression is upregulated in prostatic epithelium of intermediate- and high-grade tumours and in CRPC (Bonkhoff & Berges 2009, Celhay *et al.* 2010, Nelles *et al.* 2011). Stromal ERA expression and elevated expression of aromatase have been shown to be independent predictors of shorter time to relapse in CRPC (Celhay *et al.* 2010). Expression of the *TMPRSS2–ERG* fusion gene, which has been suggested to be a marker of an aggressive tumour phenotype found in up to 50% of prostate cancer (Qu *et al.* 2013, Razzak 2013), increased in the NCI-H660 prostate cancer cell line following treatment with an ERA agonist (Setlur *et al.* 2008). NCI-H660 is an AR-negative prostate cancer cell line expressing the *TMPRSS2-ERG* fusion gene (Mertz *et al.* 2007), derived from the lymph node metastasis of a small-cell prostate tumour with neuroendocrine differentiation (Johnson *et al.* 1989, Lai *et al.* 1995). Expression of ERA and aromatase with the R264C polymorphism has been shown to result in shorter progression-free survival and an increased risk of developing CRPC in a study of 115 men treated with docetaxel (Sissung *et al.* 2011). Taken together, these observations support the hypothesis that ERA can act as an oncogene by mediating the adverse effects of estrogen in the prostate.

Declining levels of ERB have been observed with progression from benign prostatic hyperplasia to malignant disease (Horvath *et al.* 2001), with a further decrease associated with increasing Gleason grade of prostate cancer (Leav *et al.* 2001, Asgari & Morakabati 2011, Attia & Ederveen 2012, Dey *et al.* 2013*b*). ERB expression is low in high-grade PIN of the prostate (Risbridger *et al.* 2007), reflecting its pre-malignant phenotype. It has been shown that as ERB expression declines with the development of prostate cancer, levels of HIF-1A increase, resulting in epithelial de-differentiation and growth of high-grade, aggressive tumours (Mak *et al.* 2013).

Horvath *et al.* (2001) showed in a study of 159 prostates obtained by radical prostatectomy that over 75% of tumours in their cohort did not express ERB. However, in low-grade (Gleason 3) tumours, ERB expression was maintained, and correlated positively with disease-free survival (Horvath *et al.* 2001). In an additional finding that seems to contradict these results, where ERB expression was maintained, there was a higher rate of disease relapse irrespective of tumour grade (Horvath *et al.* 2001). Other studies have demonstrated high ERB expression in bone and lymph node metastases (Zhu *et al.* 2004, Bouchal *et al.* 2011). A recent study has shown that the combination of ERB expression and AR phosphorylation in hormone-naïve prostate cancer correlates with poor clinical outcome (Zellweger *et al.* 2013). In that study, increased expression of WT ERB (ERB1) was associated with higher Gleason grade and greater proliferative activity. Fifty percent of the patients in the study cohort showed a significant increase in ERB expression with subsequent development of CRPC (Zellweger *et al.* 2013).

The variability of ERB expression in differing grades and stages of prostate cancer presents some difficulty in deciphering the underlying mechanisms and role of ERB in prostate carcinogenesis. If ERB is tumour-suppressive, then it is logical that its expression declines with advancing carcinogenesis. However, this does not explain why ERB expression is then high in lymph node or bone metastases (Zhu *et al.* 2004, Bouchal *et al.* 2011), or the observed correlation between high ERB expression and poor prostate cancer prognosis (Horvath *et al.* 2001, Zellweger *et al.* 2013). This may be due to varying levels of promotor methylation throughout the carcinogenic process introducing reversible, stage- and tissue-specific changes in ERB expression and altering its transcriptional role (Risbridger *et al.* 2007, Cotrim *et al.* 2013). In addition, it has been proposed that ERB expression may confer a selective advantage for subclones of prostate cancer cells to metastasise (Zhu *et al.* 2004), resulting in the maintenance of ERB expression in metastatic deposits. A further possible explanation for this discrepancy is variability in the specificity and sensitivity of commercially available ERB antibodies (Skliris *et al.* 2002, Hartman *et al.* 2012). Different ERB antibodies have been shown to only be suitable for particular experimental applications (Weitsman *et al.* 2006), creating some difficulty in the interpretation of results from different studies.

More recently, there is increasing evidence that expression of the ERB isoform, ERB2, is increased in high-grade and metastatic prostate cancer (Dey *et al.* 2012). ERB2 may act as an oncogene and has been implicated specifically in the process of cancer metastasis (Chen *et al.* 2009, Leung *et al.* 2010, Dey *et al.* 2012). If the antibodies used in the abovementioned studies (Horvath *et al.* 2001,

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Zhu *et al*. 2004, Bouchal *et al*. 2011, Zellweger *et al*. 2013) are in fact detecting ERB2, rather than ERB1, some of the abovementioned contradictions may be explained. Further detailed study is required to answer this definitively.

The role of ERB isoforms

At least five splice variants of ERB have been identified (Leung *et al*. 2006*b*) (Fig. 2). Expression of ERB3 is limited to the testis (Moore *et al*. 1998), but ERB1, ERB2, ERB4 and ERB5 are known to be expressed in the prostate, and there is increasing evidence indicating that ERB2 in particular acts as an oncogene in direct opposition to ERB1 (Chen *et al*. 2009). ERB1 is composed of eight exons, the first six of which are common to the five isoforms. The isoforms share the same first four functional domains with ERB1 (including the DBD), but the LBD differs (Moore *et al*. 1998, Hanstein *et al*. 1999, Leung *et al*. 2010). ERB2 and ERB5 have been studied in detail in prostate cancer and shown to correlate with poor prognosis (Leung *et al*. 2010, Dey *et al*. 2012). Specifically, co-expression of nuclear ERB2 and cytoplasmic ERB5 was shown in a study of 144 patients with long-term follow up to be an independent prognostic marker for biochemical relapse, postoperative metastasis and time to metastasis following radical prostatectomy for localised prostate cancer (Leung *et al*. 2010). While ERB2 is the dominant isoform in prostate cancer, its mechanism of action remains unclear as it lacks the LBD. ERB2 seems to act as a transcriptional repressor of ERB1, thus disabling the usual, protective effect of ERB1 (Cotrim *et al*. 2013). One hypothesis proposed by Leung *et al*. (2006*b*) is that whilst ERB1 functions as a homodimer, ERB isoforms function only when heterodimerised with ERB1. These ERB heterodimers form preferentially under the influence of oestradiol (E2) and have higher transcriptional activity than the ERB1 homodimer. Interestingly, phytoestrogens such as genistein promote formation of the ERB1 homodimer. As ERB2 lacks a LBD, it is proposed that when it is heterodimerised with ERB1, transcription is inhibited. In this model, ERB2 may therefore function as a dominant-negative regulator of ERB1 activity. This may explain one way that ERB transcription can be modulated in cell- and tissue-specific contexts (Leung *et al*. 2006*b*, Cotrim *et al*. 2013).

In ovarian carcinoma, levels of ERB5 mRNA are elevated, compared with benign tissues, suggesting it has an oncogenic role in that particular context (Suzuki *et al*. 2008). A study of stable tetracycline-inducible ERB2-expressing MCF7 breast cancer cells has suggested that ERB2 can also heterodimerise with ERA to induce ERA degradation and inhibition of ERA transcription (Zhao *et al*. 2007).

The influence of ERB2 in both prostate and breast cancer metastasis is thought to result from regulation of genes responsible for EMT (Leung *et al*. 2010, Dey *et al*. 2012, Roy *et al*. 2012, Yang *et al*. 2012). EMT is a marker of early oncological change, which enables cancer cells to

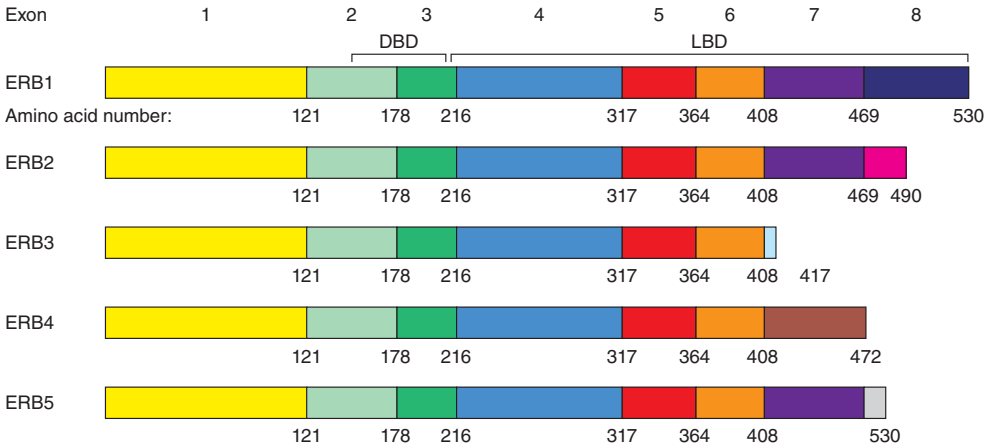

Figure 2

Schematic showing the structure of ERB isoforms. ERB1 (WT) contains eight exons, the first six of which are common to the other isoforms. All five isoforms share common DNA-binding domains (DBD), but the ligand-binding domains (LBD) differ. Data from Uniprot (http://www.uniprot.org/) and ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

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invade surrounding tissues and eventually metastasise to distant sites (Hanahan & Weinberg 2011). Although well-characterised *in vitro*, there is still some controversy as to whether the EMT programme occurs *in vivo* or is an artefact of cell line studies, principally because of difficulties differentiating transformed epithelial cells from the surrounding tumour stromal tissue, and the fact that metastatic deposits often closely resemble the epithelial tissue of origin (Hollier *et al.* 2009). Nevertheless, evidence is emerging that EMT markers can be observed in circulating tumour cells, suggesting that it does occur *in vivo* (Li *et al.* 2013).

In a study conducted in prostate cancer cell lines, expression of ERB2 was shown to result in upregulation of the EMT genes *TWIST1* (which correlates with high-grade prostate cancer) and *RUNX2* (normally repressed by ERB1) (Dey *et al.* 2012). This interaction between ERB2 and EMT genes is facilitated by a proto-oncogene, PELP1, which interacts with a number of steroid hormone receptors including ERA (Vadlamudi *et al.* 2001), ERB and AR (Yang *et al.* 2012). In breast cancer, PELP1 has been shown to interact with histones to remodel chromatin and modulate expression of key EMT genes such as *TWIST1*, *SNAIL* and *ZEB* (Roy *et al.* 2012). It is therefore possible that PELP1 and ERB2 have a role in the promotion of prostate cancer metastasis. If correct, this hypothesis could explain the previously discussed findings of high ERB expression in bone and lymph node metastases, and the correlation between persistent ERB expression in high-grade prostate cancer and greater risk of disease relapse (Horvath *et al.* 2001, Zhu *et al.* 2004, Bouchal *et al.* 2011, Zellweger *et al.* 2013). This is an important area for further investigation.

The role of ER-mediated inflammation in mechanisms of cancer progression

Inflammation is a well-established process in the development and progression of cancer (Hanahan & Weinberg 2011) (Fig. 3). Several inflammatory mechanisms, centering on ER function, have been implicated in the development and progression of prostate cancer. Loss of E-cadherin is a well-established marker of EMT, with associated loss of cell adhesion and a resulting increase in cell motility (Guarino *et al.* 2007, Grubisha & Defranco 2013). ERB has been shown to be a negative regulator of inflammatory processes (Harris *et al.* 2003) and in the prostate its expression is known to correlate with E-cadherin levels. One hypothesis is that as ERB expression declines during the progression from benign to low-grade,

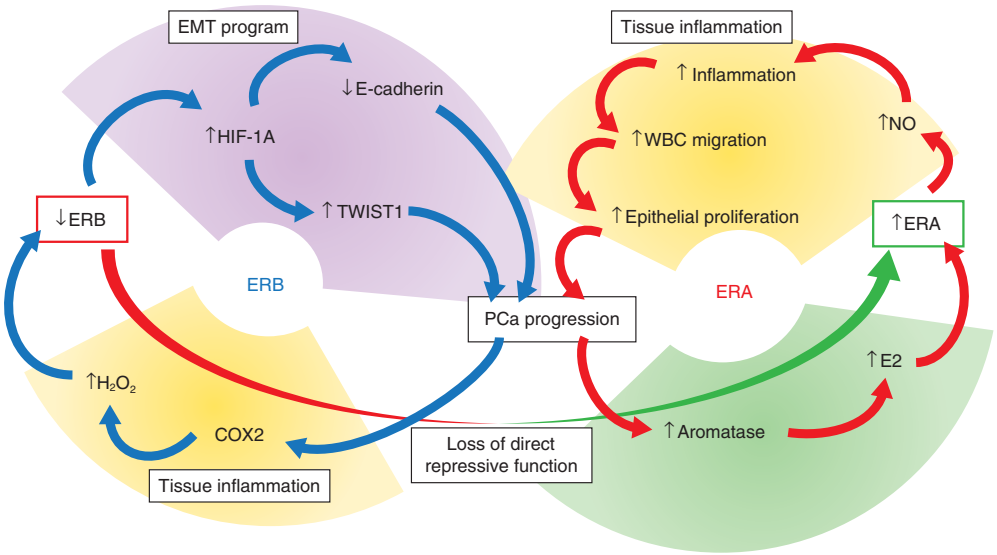

Figure 3

Proinflammatory positive feedback loops in prostate cancer progression, centering on ERB and ERA function. In response to tissue inflammation, decreased ERB expression results in upregulation of EMT programme genes leading to prostate cancer metastasis. Tissue inflammation leads to increased aromatase expression and increased inflammatory-cell epithelial infiltration. Decreased ERB transcription results in loss of direct repression of ERA. WBC, white blood cell; E2, oestradiol; NO, nitric oxide; H_2O_2, hydrogen peroxide; COX2, cyclo-oxygenase-2; PCa, prostate cancer.

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to high-grade cancer (Leav *et al.* 2001, Celhay *et al.* 2010, Asgari & Morakabati 2011), the resulting decline in E-cadherin leads to an increased propensity to develop metastatic disease. ERB transcriptional activity is sensitive to oxidation resulting from tissue inflammatory processes and a local paracrine signalling network (Grubisha *et al.* 2012). When ERB is oxidised by H_2O_2 and other reactive oxygen species, DNA binding is lost and expression of E-cadherin is reduced. The pro-inflammatory enzyme COX2, expressed by prostatic stroma, generates sufficient H_2O_2 to inactivate ERB. As COX2 is overexpressed in prostate cancer, a pro-inflammatory positive feedback loop is established (Grubisha & Defranco 2013).

ERA and aromatase also play a critical role in tissue inflammation in prostate cancer and expression of these two factors in tumour cells is an independent predictor of time to biochemical relapse (defined as two consecutive rises in serum PSA) in men treated with ADT (Celhay *et al.* 2010). In prostate cancer, expression of aromatase is increased, particularly in epithelial cells resulting in increased levels of intraprostatic estrogen, which acts via ERA to promote tissue inflammation via local generation of nitric oxide (Pinzone *et al.* 2004, Ellem & Risbridger 2007, Risbridger *et al.* 2007, Celhay *et al.* 2010, Nelles *et al.* 2011). Pro-inflammatory mediators such as TNFA and prostaglandin E_2 in turn upregulate *CYP19* expression, resulting in increased aromatase activity (Subbaramaiah *et al.* 2011). Thus an additional pro-inflammatory positive feedback loop centering on ERA function is established (Ellem & Risbridger 2007). Evidence of neutrophil and leucocyte migration from the stroma to the epithelium in mouse models confirms the presence of this inflammatory process (Bianco *et al.* 2002, 2006). Inflammatory cytokines released by the migrating immune cells result in abnormal proliferation of prostate epithelium and increase the risk of further premalignant change (Bianco *et al.* 2006).

A change in perspective: an oncogenic role for ERB?

ERB1 has been implicated directly as an oncogene (Yang *et al.* 2012). In that study, ERB1-mediated, non-androgenic AR signalling was demonstrated in several prostate cancer cell lines in hormone-deplete (castrate) conditions (Fig. 4). In the presence of DHT, AR binds to androgen-responsive elements (AREs) on DNA to initiate AR-dependent transcription. In these conditions, ERB1 and PELP1 form a complex in the nucleus. However, in the absence of DHT and with addition of E2, the ERB1–PELP1 complex binds

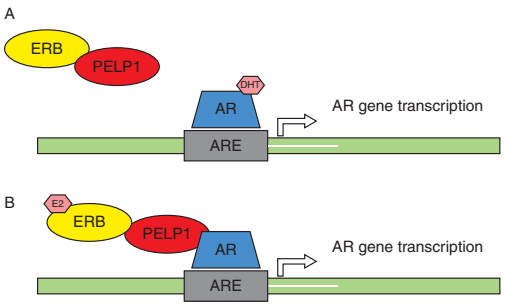

Figure 4
A hypothesis of non-androgenic, ERB-mediated transcription of AR-dependent genes via interaction with PELP1, as proposed by Yang *et al.* (2012). (A) In the presence of DHT ERB-PELP1 forms a complex, which does not bind to AR. (B) In the absence of DHT, and with oestradiol (E2) treatment, the ERB-PELP1-AR complex binds to the androgen-responsive element (ARE) to initiate AR-dependent gene transcription.
Figure adapted from Yang L, Ravindranathan P, Ramanan M, Kapur P, Hammes SR, Hsieh JT & Raj GV 2012 Central role for PELP1 in nonandrogenic activation of the androgen receptor in prostate cancer. *Molecular Endocrinology* **26** 550–561.

to AR (with PELP1 acting as a bridge between the two nuclear receptors) and is recruited to an ARE, resulting in the transcription of AR-dependent genes. This ERB1–PELP1–AR complex was shown to facilitate cellular proliferation in response to E2 treatment, demonstrating a clear mechanism by which estrogens might continue to drive prostate cancer growth and progression in the castrate environment, thereby highlighting the potential oncogenic role of ERB1. Furthermore, it has been proposed that ERB1 may have a role in mediating the 'switch' from hormone-sensitive prostate cancer to CRPC (Zellweger *et al.* 2013). It may be that ERB is only tumour-suppressive in early stages of the disease until, by means of a currently unknown mechanism, it subsequently becomes an oncogene. This is an important question for detailed investigation, as therapeutic silencing of such a 'switch' could theoretically reduce the risk of developing CRPC.

Our understanding of the role of ERB in the development and progression of prostate cancer is evolving, but there are many unresolved issues. Given the divergent activity of ERB isoforms and the potential for an oncogenic role for ERB1 (Yang *et al.* 2012), we can no longer hold to the classical paradigm of estrogen signalling in the prostate, which surmises that ERA is tumour promoting and ERB is tumour suppressive. In order to progress our understanding of estrogen biology in cancer it is critical that the mechanisms underlying the differential functions of ERA, ERB and the various ERB isoforms are elucidated in detail (Madak-Erdogan *et al.* 2013). Recent advances in

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understanding the function of the two ERs at the genomic level have been beginning to provide insights into this complex area. Madak-Erdogan *et al.* (2013) used ChIP-seq to demonstrate the genome-wide chromatin binding profiles and differing transcriptional responses of ERA or ERB in the MCF7 breast cancer cell line. Their data confirmed the previous finding using the tagged ERB ChIP-seq approach (Zhao *et al.* 2010) demonstrating significant overlap between the binding sites of ERA and ERB. Specifically, when each of the receptors was present alone, there was a 40% overlap between ERA and ERB DNA-binding sites. However, when co-expressed, the number of binding sites available for each ER dropped by ~50%. This suggests a complex mechanism where each ER restricts the total number of binding sites available to the other, but when the activity of one ER is reduced, chromatin binding by the other is increased. While the functional consequences of altered ERA and ERB chromatin binding in these different contexts remain to be fully elucidated, the proliferative effects of estrogen acting via ERA were reduced with the co-expression of ERB. When expressed in isolation, ERA regulates cell cycle genes, helping push cells from growth-arrested states into DNA synthesis and subsequent mitosis. However, when ERB is co-expressed, ERA's ability to respond to ligand is reduced and cell proliferation decreases. ERB's anti-proliferative function was demonstrated to occur through direct binding to apoptosis and cell-cycle regulation genes (Madak-Erdogan *et al.* 2013). Similar results were demonstrated by Le *et al.* (2013) in ChIP-seq of MCF7/C4-12 cells (derivative of MCF7 with no ERA expression) transfected to stably express ERB. These data support the previously discussed hypothesis that ERB is tumour-suppressive and a negative regulator of ERA, functioning in a variable manner according to the particular cellular context (Zhao *et al.* 2010, Bottner *et al.* 2014, Cotrim *et al.* 2013). However, as these data were generated using breast cancer cell lines, it is important that the hypothesis is further tested in appropriate prostate cancer models. The isoform specificities of the antibodies used in the ChIP-seq study (Madak-Erdogan *et al.* 2013) are unknown and therefore it is not clear how these findings in MCF7 cells are applicable to the previously discussed differential functions of ERB isoforms (Leung *et al.* 2010, Dey *et al.* 2012). Clearly, there is an urgent need to develop specific antibodies to ERB and its isoforms to address these questions (Haldosen *et al.* 2014).

Conclusions

Despite a number of promising preclinical studies showing efficacy of ERB-selective agents in prostate cancer (Kim *et al.* 2002*a*,*b*, McPherson *et al.* 2010), there is currently no evidence of clinical benefit from the use of these treatments in terms of disease-specific or overall survival. The underlying reasons for this necessitate further investigation. One possibility is insufficient dosing of the therapeutic agents in question (Chadha *et al.* 2008). Most of the preclinical studies of ERB-selective agents and much of our current knowledge of ERB biology results from studies conducted in various prostate cancer cell lines. The expression profiles of the nuclear receptors AR, ERA and ERB vary between each of the commonly used cell lines and different authors report contrasting results in individual cell lines (Table 1) (Veldscholte *et al.* 1990, Kim *et al.* 2002*b*, Holbeck *et al.* 2010, Nakajima *et al.* 2011). None of these commonly used cell lines are entirely representative of human tissue, as exemplified by the fact that in the human prostate ERA expression is predominantly stromal, whereas luminal epithelial cells express ERB and AR, and basal epithelial cells only express ERB (Ruizeveld de Winter *et al.* 1991, Bonkhoff & Berges 2009). Cell line models, therefore, cannot reproduce the stromal–epithelial interactions known to be important in cancer development and progression (Hanahan & Weinberg 2011), or the complex interplay that has been observed between ERA and ERB, and how transcription from activation of one receptor impacts the availability of DNA-binding sites to the other (Madak-Erdogan *et al.* 2013). Studies conducted in *ex vivo*

Table 1 Variability of reported nuclear receptor expression in commonly used prostate cancer cell lines

References	Cell line	AR	ERA	ERB
Veldscholte *et al.* (1990) and Kim *et al.* (2002*a*,*b*)	LNCaP	Positive (mutant)	Negative	Positive
Nakajima *et al.* (2011)	DU145	Negative	Negative	Positive
Holbeck *et al.* (2010)	DU145	Negative	Negative	Negative
Holbeck *et al.* (2010) and Nakajima *et al.* (2011)	PC3	Negative	Positive	Positive

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primary human tissue culture (Centenera *et al.* 2012) or xenografts of human tumours (Lawrence *et al.* 2013) may be helpful in this regard.

Estrogen-related pathways are clearly of great importance in the development and progression of hormone-dependent cancers such as prostate cancer, but the role of ERB remains controversial, with numerous contradictions in the published literature. Our current understanding of ER biology in the prostate is insufficient to facilitate precise manipulation of the molecular machinery in a meaningful fashion (Abd Elmageed *et al.* 2013). Recent developments in the understanding of apparently opposing ERB isoforms (Leung *et al.* 2010) and the mechanisms governing ERB transcription are beginning to provide greater insights into ERB biology with implications not just for prostate cancer but also for colon, breast and ovarian cancers (Suzuki *et al.* 2008, Chantzi *et al.* 2013, Dey *et al.* 2013*a*). In order to determine whether ERB represents a useful therapeutic target in prostate cancer, and more specifically in CRPC, it is vital that these mechanisms are fully elucidated. Given that ERA and ERB can homo- or heterodimerise with ERB isoforms, the cross-reactivity between different estrogenic ligands, the differing effects of ERB in specific cellular contexts, and the fact that ERA and ERB can recognise the same DNA-binding sites and interact with common co-regulators, this is likely to be a difficult task (Shaaban *et al.* 2003, Zhao *et al.* 2010, Cotrim *et al.* 2013, Le *et al.* 2013, Madak-Erdogan *et al.* 2013). The challenge will be to identify and characterise the ERA- and ERB-unique DNA-binding sites, and furthermore, to define the ERB isoform-specific DNA binding sites in order to determine their respective functions. To improve outcomes for patients, there is an urgent need for detailed understanding of the mechanisms governing the differential functions of the two ERs in tissue- and disease-specific contexts as well as investigation of novel therapeutic agents that selectively target ERA- and ERB-dependent pathways.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

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Author contribution statement
A W Nelson and W D Tilley conceptualised and designed the structure of the article. A W Nelson conducted the literature review. A W Nelson and W D Tilley co-wrote the manuscript. D E Neal and J S Carroll provided critical review and revision of the manuscript.

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Comprehensive assessment of estrogen receptor beta antibodies in cancer cell line models and tissue reveals critical limitations in reagent specificity

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ABSTRACT

Estrogen Receptor-β (ERβ) has been implicated in many cancers. In prostate and breast cancer its function is controversial, but genetic studies implicate a role in cancer progression. Much of the confusion around ERβ stems from antibodies that are inadequately validated, yet have become standard tools for deciphering its role. Using an ERβ-inducible cell system we assessed commonly utilized ERβ antibodies and show that one of the most commonly used antibodies, NCL-ER-BETA, is non-specific for ERβ. Other antibodies have limited ERβ specificity or are only specific in one experimental modality. ERβ is commonly studied in MCF-7 (breast) and LNCaP (prostate) cancer cell lines, but we found no ERβ expression in either, using validated antibodies and independent mass spectrometry-based approaches. Our findings question conclusions made about ERβ using the NCL-ER-BETA antibody, or LNCaP and MCF-7 cell lines. We describe robust reagents, which detect ERβ across multiple experimental approaches and in clinical samples.

1. Introduction

Estrogen receptor beta (ERβ) was first discovered in the rat prostate (Kuiper et al., 1996). Since then, there has been considerable interest in understanding its role in both breast and prostate cancer. Despite a large body of literature, the function of ERβ in these two cancers remains unclear (Haldosen et al., 2014; Nelson et al., 2014). Most authors agree that ERβ has a predominantly antiproliferative, pro-apoptotic and tumor-suppressive role (Attia and Ederveen, 2012; Bottner et al., 2014; Chang and Prins, 1999; Ellem and Risbridger, 2007; Horvath et al., 2001; Madak-Erdogan et al., 2013; McPherson et al., 2010; Muthusamy et al., 2011; Nakajima et al., 2011; Rizza et al., 2014; Ruddy et al., 2014; Zhu et al., 2004), however ERβ has also been implicated as an oncogene. This is particularly in the context of Castrate Resistant Prostate Cancer (CRPC) where it has been proposed as a driver of androgen receptor (AR)-dependent gene transcription (Yang et al., 2012, 2015), along with a potential role in mediating the transition from hormone-sensitive to CRPC (Zellweger et al., 2013). In breast cancer, it has been suggested that ERβ may have a 'bi-faceted role' and should not simply be considered a tumor-suppressor (Jonsson et al., 2014). ERβ has been reported to 'cross-talk' with androgen receptor-positive breast cancer (Rizza et al., 2014) and may be an important factor in ERα-negative breast cancer (Gruvberger-Saal et al., 2007; Smart et al., 2013).

Inconsistencies in the reported expression of ERβ in breast and

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prostate cancers as determined by immunohistochemistry (IHC) have contributed to this uncertainty. In prostate, most data support the conclusion that ERβ is highly expressed in benign epithelial cells, with expression declining in cancer development and inversely correlating with increasing Gleason grade (Asgari and Morakabati, 2011; Attia and Ederveen, 2012; Dey et al., 2014; Horvath et al., 2001; Leav et al., 2001; Risbridger et al., 2007). However, it has also been reported that ERβ expression is high in bone and lymph node metastases (Bouchal et al., 2011; Zhu et al., 2004) and that high ERβ expression correlates with poor clinical prognosis (Horvath et al., 2001; Zellweger et al., 2013). In breast cancer, high ERβ expression has been described both as a poor (Guo et al., 2014) and favorable (Esslimani-Sahla et al., 2004; Gruvberger-Saal et al., 2007; Hieken et al., 2015; Leygue and Murphy, 2013; Myers et al., 2004; Omoto et al., 2002; Roger et al., 2001) prognostic marker, with others finding no association between clinico-pathological parameters and ERβ expression (Umekita et al., 2006).

It is recognized that there is wide variability in the sensitivity and specificity of ERβ antibodies, which may contribute to the uncertainties surrounding its molecular action and tissue expression (Choi et al., 2001; Hartman et al., 2012; Skliris et al., 2002; Weitsman et al., 2006; Wu et al., 2012). Previous ERβ antibody validation studies have been published (Carder et al., 2005; Choi et al., 2001; Skliris et al., 2002; Weitsman et al., 2006; Wu et al., 2012), however some of them are limited by reliance on two key assumptions. Firstly, that when assessing an antibody by Western blotting in a cell line model, the factor of interest is expressed and secondly, when assessing an antibody's specificity by IHC in tissue, the tissue expression of the factor has been well characterized. In the case of ERβ, these assumptions are problematic, as its expression in commonly used cell line models and in tissues is not universally accepted (Al-Bader et al., 2011; Asgari and Morakabati, 2011; Attia and Ederveen, 2012; Bouchal et al., 2011; Dey et al., 2014; Gruvberger-Saal et al., 2007; Guo et al., 2014; Hieken et al., 2015; Holbeck et al., 2010; Horvath et al., 2001; Leav et al., 2001; Nakajima et al., 2011; Omoto et al., 2002; Risbridger et al., 2007; Shaaban et al., 2003; Skliris et al., 2002; Umekita et al., 2006; Zellweger et al., 2013; Zhou et al., 2012; Zhu et al., 2004).

In light of this, we sought to test and validate six commonly used, commercially available ERβ antibodies and two non-commercially available ERβ antibodies (Choi et al., 2001; Wu et al., 2012) in a systematic manner that addresses these assumptions. To achieve this, we employed a number of assays for antibody validation, including a novel proteomic-based pull down method called Rapid Immunopreciptation Mass spectrometry of Endogenous protein (RIME) (Mohammed et al., 2013). We then applied successfully validated antibodies to cell line models of breast and prostate cancer commonly used for studies of ERβ to assess them for ERβ expression. ERβ expression in the cell lines was validated by a non-antibody dependent, targeted proteomics method known as Parallel Reaction Monitoring (PRM) (Gallien et al., 2012). Finally, benign and malignant prostate and breast tissues were stained with the validated ERβ antibody to assess tissue expression of ERβ by IHC.

2. Materials and methods

2.1. Cell culture

The cancer cell line MDA-MB-231 with doxycycline-inducible ERβ expression (MDA-MB-231-ERβ) (Reese et al., 2014) was cultured in Dulbeccos Modified Eagle Medium with F12 supplement (DMEM/F12) with 10% heat-inactivated tetracycline-free fetal bovine serum (FBS) (Fisher-Scientific), 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, 5 μg/ml blasticidin S (Invivogen) to select for the tetracycline repressor and 500 μg/ml zeocin (Invitrogen) to select for the ERβ expression vector. To induce ERβ expression in MDA-MB 231-ERβ cells, 15 cm^2 plates were seeded with 5×10^6 cells and doxycycline added at either 0.1 μg/ml (for Western blot, real-time polymerase chain reaction (qRT-PCR) and PRM) or 0.5 μg/ml (for RIME) for 24 h. The MCF-7 breast cancer cell line was cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% heat-inactivated FBS (Fisher-Scientific), 2 mM L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin. The LNCaP prostate cancer cell line was cultured in RPMI 1640 with 10% heat-inactivated FBS (Fisher-Scientific), 2 mM L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin. All cells were incubated at 37 °C with 5% CO2 and cultured to 80–90% confluence. LNCaP and MCF-7 cell lines were obtained from ATCC (Middlesex, UK) and validated by STR genotyping.

2.2. Preparation of mRNA and qRT-PCR

MDA-MB-231-ERβ+, MDA-MB-231-ERβ–, MCF-7 and LNCaP cells were harvested for collection of mRNA using the RNEasy Mini Kit (Qiagen, California USA). On-column DNase digestion was performed to remove contaminating genomic DNA. RNA was quantified with the NanoDrop 8000 (Thermo Scientific, Delaware USA). Samples containing 250 ng random primers, 1 μg RNA, 1 μl 10 mM dNTP mix and water to a total volume of 13 μl were heated to 65 °C for 5 min, followed by 1 min incubation on ice. To each sample 4 μl 5X First-strand buffer, 1 μl 0.1 M DTT, 1 μl RNaseOUT and 1 μl SuperScript III reverse transcriptase (RT) (Thermofisher Scientific, Leicestershire, UK) were added and incubated at 25 °C for 5 min then 50 °C for 60 min followed by heating at 70 °C for 15 min qRT-PCR primers for wild type ERβ (Table 1) were designed based on published sequence of *ESR2* (available from USCS genome browser at http://genome.ucsc.edu/) using the Primer3 software package (Koressaar and Remm, 2007; Untergasser et al., 2012) available at http://bioinfo.ut.ee/primer3-0.4.0/primer3/. UBC primers (SY121212648) were obtained from Sigma-Aldrich (Dorset, UK). Each qRT-PCR reaction contained 7.5 μl Power SYBR Green PCR Master Mix (Applied Biosystems, California USA), 0.5 μl of 10 μM primer mix, 2 μl of a 1:5 dilution of cDNA and nuclease-free water to a final volume of 15 μl. Reactions were performed with the Stratagene Mx3005P RealTime machine in triplicate. Hot-start Taq polymerase was heat-activated at 95 °C for 10 min followed by 40 cycles of 15 s at 95 °C and 30 s at 60 °C. Fluorescence was read in each cycle and a melting curve constructed as the temperature was increased from 65 °C to 95 °C with continuous fluorescence readings. UBC was used as a control gene to normalize between the samples and relative expression determined using the delta-delta Ct method (Livak and Schmittgen, 2001).

2.3. Western blotting

MDA-MB-231-ERβ+, MDA-MB-231-ERβ–, MCF-7 and LNCaP cells were harvested for nuclear extract using the Ne-Per nuclear extraction kit (Thermo Scientific Pierce, Rockford IL USA) according to the manufacturer's instructions. Extracted protein was quantified using the Direct Detect system (Merrick Millipore, Massachusetts USA). Nuclear extracts were prepared with 4X protein sample loading buffer (LI-COR Biosciences, USA), 10X NuPage sample reducing agent (Thermofisher Scientific, Leicestershire, UK) and water, and 15 μg protein per lane loaded into Bolt 4–12% Bis-Tris gels (Thermofisher Scientific, Leicestershire, UK). Gels were run with MOPS running buffer for 30 min at 60 V followed by 30 min at 120 V. Western transfer was performed using the iBlot system (Invitrogen, Paisley, UK) according to the manufacturer's

instructions. Odyssey blocking buffer (LI-COR Biosciences, USA) was added to membranes for one hour at room temperature. Primary antibodies (Table 2 and Supplementary Fig. 1) were added at the following dilutions and incubated overnight at 4 °C: Novocastra-ER-beta (EMR02-NCL-ER-BETA) (Leica Biosystems, Newcastle, UK) 1:100, ERβ1 PPG5/10 (MAI-81281) (Thermo Scientific Pierce, Rockford IL USA) 1:100, ERβ-antibody H150 (sc8974) (Santa Cruz Biotechnology, Dallas TX, USA) 1:200, CWK-F12, USA) (Choi et al., 2001) 1:200, MC10 (Wu et al., 2012) 1:300, GeneTex ERβ 70182 (Irvine, CA, USA) 1:200, ERβ 06-629 (Merck Millipore, Watford, UK), 1:500, Abcam 288 [14C8] (Cambridge, UK) 1:500. The following were used as loading controls: rabbit anti-beta actin (ab8227) (Abcam, Cambridge, UK) 1:5000 or mouse anti-beta actin [AC-15](ab6276) 1:1000 according to the species of the ERβ antibody. The membranes were washed three times with PBS/0.1% tween and incubated with secondary antibodies for one hour at room temperature: Goat anti-mouse (green) 1:5000 with Goat anti-rabbit (red) 1:20000 or Goat anti-rabbit (green) 1:5000 with Goat anti-mouse (red) 1:20000 according to the species of the ERβ antibody. Membranes were imaged using the Li-Cor Odyssey fluorescent imaging system (LI-COR Biosciences, USA).

2.4. Immunohistochemistry (IHC)

Formalin-fixed, paraffin-embedded MDA-MB-231-ERβ− and MDA-MB-231-ERβ+ cell pellets were generated, with ~2 × 10^7 cells per pellet. ERβ expression was induced with 0.5 μg/ml doxycycline for 24 h. Antigen retrieval was achieved by incubating in citrate-based retrieval solution for 20 min. Sections were stained using CWK-F12 ERβ antibody, diluted 1:250 in standard Bond diluent using Leica's Polymer Refine Kit (Catalogue No: DS9800) on the automated Bond platform (Leica Biosystems Newcastle Ltd, Newcastle UK). Images were captured using Aperio® software (Leica Biosystems Newcastle Lt, Newcastle UK).

A prostate tissue microarray (TMA) was created from a random selection of prostate cancers, including a range of different tumor grades, and benign prostatic tissue (10 cancer, 5 benign in total) (ethical approval: ProMPT study MREC/01/4/061). The areas to be sampled from the formalin-fixed and paraffin embedded tissue blocks were marked on the corresponding Haematoxylin and Eosin stained paraffin sections. Each block was assessed to ensure that there was an adequate amount of tissue for sampling, and cores of tissue punched from the selected area of the block using 5 mm skin biopsy punches. Each core was re-embedded into a new recipient paraffin block and its position in the block recorded on a TMA map. Cores of pig kidney were used as orientation markers.

The breast TMA was constructed using the Chemicon Advanced Tissue Arrayer (Merck Millipore, Germany) according to the manufacturer's instructions. This contained 30 benign samples, 56 grade I, 55 grade II and 57 grade III ER alpha positive tumors. An additional TMA was constructed from 10 invasive carcinomas and 10 non-malignant tissues for optimisation of antibody staining. To ensure adequate representation of the tissue, core size of 1 mm was selected and cores arranged in duplicate with liver and spleen as orientation cores. The study protocol for tissue collection was approved by the University of Adelaide Human Research Ethics Committee (#s H-2005-065).

For the prostate IHC, 3.5 μm sections were cut and mounted onto charged slides, dried and sealed with paraffin. The CWK-F12 ERβ antibody was further optimized to the clinical samples and diluted at 1:200 in diluent consisting of 1% donkey serum, 0.05% Tween20 in 300 mM TBS to reduce background staining. Antigen retrieval was achieved by incubating in Tris EDTA for 20 min at 100 °C. Images were captured at 250 × magnification using Image Pro-Insight (Media Cybernetics. Rockville, MD. USA).

For the breast IHC, 4 μm sections were cut and adhered to Superfrost UltraPLUS slides (Thermo-Fisher Scientific #1014356190). Slides were dewaxed in xylene followed by 100% EtOH and then PBS. Endogenous peroxidase was quenched with 0.3% hydrogen peroxide (Ajax Finchem ##7722-84-1). Antigen retrieval was performed in 10 mM Citric acid buffer (pH 6.0) within a decloaking chamber (Biocare Medical #DC2012), for 5 min at 120 °C. Slides were blocked in 5% normal goat serum (Sigma-Aldrich #G9023) in PBS for 30 min at room temperature. CWK-F12 antibody was added at a dilution of 1:100 and incubated overnight at 4 °C. A second section of TMA tissue that received buffer in the absence of primary antibody served as a negative control. Secondary antibody (biotinylated anti-mouse antibody (Dako #E0433) diluted in PBS with 5% normal goat serum was added and incubated for 60 min at room temperature. Sections were washed twice in PBS followed by addition of HRP-conjugated streptavidin (Dako #P0397). Tissue was counterstained with haematoxylin and mounted under DPX mountant (Sigma #06522). Slides were scanned on a Nanozoomer slide scanner (Hamamatsu #C9600).

2.5. Rapid immunoprecipitation and Mass Spectrometry of Endogenous Protein (RIME)

RIME experiments were conducted as previously described (Mohammed et al., 2013). Briefly, MDA-MB-231-ERβ+, MDA-MB-231-ERβ− (2 × 10^7 cells per condition for antibody evaluation), LNCaP and MCF-7 cells (4 × 10^7 cells per condition for cell line characterization) were grown in 15 cm^2 plates to 90% confluency. Cells were crosslinked with media containing 1% EM grade formaldehyde (TEBU biosciences, Peterborough UK) for 8 min and the formaldehyde quenched with 0.1 M glycine. Cells were washed, harvested and pelleted in cold PBS. To enrich the nuclear fraction the cell pellet was suspended in 10 ml of lysis buffer 1 (50 mM HEPES-KOH [pH 7.5], 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP-40 or Igepal CA-630, and 0.25% Triton X-100) for 10 min at 4 °C. Cells were pelleted and resuspended in lysis buffer 2 (10 mM Tris-HCL [pH 8.0], 200 mM NaCl, 1 mM EDTA, and 0.5 mM EGTA) for five minutes at 4 °C. Cells were pelleted and resuspended in 300 μl of lysis buffer 3 (10 mM Tris-HCl [pH 8], 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.1% Na-deoxycholate, and 0.5% N-lauroylsarcosine) and sonicated (Diagenode bioruptor. Diagenode, Seraing Belgium) for 45 min 30 μl of 10% Triton-X was added and the sonicated lysate centrifuged at 17,000G for 10 min to remove cell debris. The supernatant was incubated with 100 μl of magnetic beads (Dynabeads®, Thermo Fisher Scientific, Waltham MA USA) pre-bound with antibody.

For evaluation of the 8 ERβ antibodies, immunoprecipitations (IP) were set up each for MDA-MB-231-ERβ− and MDA-MB-231-ERβ+ cells using 10 μg of antibody (NCL-ER-BETA, GeneTex 70182, Millipore 06-629, Abcam 288 [14C8], MC10, CWK-F12, sc8974 and PPG5/10). For characterization of LNCaP and MCF-7 cells, 20 μg of MC10 ERβ antibody was used in each IP. In all cases, 10 μg of E2F1-C20 IP was used as a positive control (Sc-193, Santa Cruz Biotechnology, Dallas TX, USA) and species-specific IgG used to detect non-specific pull-down (Mouse sc2025 or Rabbit sc2027, Santa Cruz Biotechnology, Dallas TX, USA). Samples were incubated

Table 1
Sequence of ERβ mRNA primers used in qRT-PCR validation of the MDA-MB-231-ERβ cell line. These primer sequences flank a region spanning exons 2 and 3, which is common to wild type ERβ and ERβ isoforms.

Primer	Sequence
ERβ – fwd	5′ AAAACCGGCGCAAGAGCTG 3′
ERβ – rev	3′ TGCTCGTCGGCACTTCTCTG 5′

Table 2
Details of ERβ antibodies validated. Application details are as recommended by the manufacturer. IHC, immunohistochemistry; WB, western blot; IF, immunofluorescence; ChIP, chromatin immunoprecipitation; ELISA, enzyme-linked immunosorbent assay; Flow cyt, flow cytometry; ICC, immunocytochemistry; IP, immunoprecipitation; Wt, wild type; NTD, N terminal domain; LBD, ligand binding domain.

Antibody	Immunogen	Host species	Class	Binding region	Application
NCL-ER-BETA	Recombinant protein. Wt ERβ. C terminus	Mouse	Monoclonal	C terminus	IHC, WB
PPG5/10	Synthetic peptide C terminus of wt ERβ	Mouse	Monoclonal	C terminus	IF, IHC, WB
GeneTex 70182	Amino acids 1-153 of human ERβ expressed in E.coli	Mouse	Monoclonal	N terminus	IP, WB, ChIP
Millipore 06-629	Amino acids 46-63 of human ERβ	Rabbit	Polyclonal	NTD	WB, IHC
Santa cruz sc8974	Amino acids 1-150 of human ERβ	Rabbit	Polyclonal	N terminus	WB, ChIP, IF, ELISA
Abcam 288 [14C8]	Recombinant fusion protein. Amino acids 1-153 of human ERβ in E.coli	Mouse	Monoclonal	N terminus	WB, Flow cyt, IHC, ICC, ChIP
CWK-F12	Recombinant protein. Amino acids 272-285 of human wt ERβ	Mouse	Monoclonal	LBD	WB, IP, IHC
MC10	Fusion protein. Amino acids 1-140 of human ERβ in E.coli	Mouse	Monoclonal	N terminus	IHC, IP, WB, IF

overnight at 4 °C. Beads were washed 10 times in 1 ml RIPA buffer (50 mM HEPES pH 7.6, 1 mM EDTA, 0.7% Na deoxycholate, 1% NP-40, 0.5 M LiCL) and twice in 100 mM ammonium hydrogen carbonate (AMBIC) solution. Dry, frozen beads were submitted for tryptic digestion of bead-bound protein, and peptides pulled down by IP identified by mass-spectrometry (LTQ Velos-Orbitrap MS, Thermo Fisher Scientific, Waltham MA USA). Raw MS data files were processed using Proteome Discoverer v.1.3 (Thermo Scientific). Processed files were searched against the SwissProt human database using the Mascot search engine version 2.3.0 with a false discovery rate (FDR) of less than 1%. For each ERβ antibody tested, the resulting list of purified peptides identified was filtered against the corresponding IgG control to remove non-specific proteins pulled down. Mean percentage ERβ peptide coverage, and mean number of unique ERβ peptides identified in biological duplicate experiments were calculated.

2.6. Parallel Reaction monitoring (PRM)

Nuclear pellets of MDA-MB-231-ERβ+, MDA-MB-231-ERβ−, LNCaP and MCF-7 cells were prepared using the Panomics nuclear extraction kit (Affymetrix, CA USA) as per the manufacturer's provided instructions. Nuclear pellets were lysed in 8 M Urea, 0.1% SDS in 50 mM TEAB by sonication twice, each for 5 min. After protein estimation 20 μg of protein was taken for tryptic digestion. 50 mM of TEAB (pH = 8) was added up to a total volume of 100 μl. Cysteines were reduced in 0.1 mM DTT for 1 h at room temperature and alkylated in 0.1 mM IAA for 30 min at room temperature in the dark. Alkylation was quenched by adding 0.1 mM DTT for 15 min. Trypsin (Promega trypsin (V5111)) was added in a 1:100 trypsin:-protein ratio for 1 h at room temperature. Another batch of trypsin (1:100 ratio) was added to have a final ratio of 1:50 for incubation overnight. Samples were acidified to a final concentration of 1% formic acid (FA) and cleaned over C18 spin columns (Harvard apparatus C18 Micro SpinColumn™). After elution from the columns samples were lyophilized in a speedvac and resolubilized in 0.1% FA, 5% ACN to a final peptide concentration of 1 μg/μl. Samples were subjected to liquid chromatography-electrospray ionization in an Orbitrap nano-ESI Q-Exactive mass spectrometer (Thermo Scientific), coupled to a nanoLC (Dionex Ultimate 3000 UHPLC). Samples were trapped on a 100 μm × 2 cm, C18, 5 μm, 100 trapping column (Acclaim PepMap 100) in μL-pickup injection mode at 4 μL/min flow rate for 10 min. Samples were loaded on a Rapid Separation Liquid Chromatography, 75 μm × 25 cm nanoViper C18 3 μm 100 column (Acclaim, PepMap) retrofitted to an EASY-Spray source with a flow rate of 300 nL/min (buffer A, HPLC H_2O, 0.1% FA; buffer B, 100% ACN, 0.1% FA; 60-min gradient; 0–5 min: 5% buffer B, 5–45 min: 5 to >56% buffer B, 45.1–50 min: 56% to >95% buffer B, 50.1–60 min, 5% buffer B). Peptides were transferred to the gaseous phase with positive ion electrospray ionization at 1.8 kV. Precursors were targeted in a 2Th selection window around the m/z of interest. Precursors were fragmented in high-energy collisional dissociation mode with normalized collision energy dependent on the target peptide. The first mass analysis was performed at a 70,000 resolution, an automatic gain control target of 3×10^6, and a maximum C-trap fill time of 200 ms; MS/MS was performed at 35,000 resolution, an AGC target of 5×10^4, and a maximum C-trap fill time of 100 ms. Spectra were analyzed using Skyline with manual validation.

2.7. Statistics

Differences in ERβ mRNA levels observed in MDA-MB-231-ERβ− and MDA-MB-231-ERβ+ conditions were analyzed using unpaired t-tests. Differences were considered statistically significant at $p \leq 0.05$. Data presented are mean of technical triplicate experiments ± standard deviation. Analysis was performed in GraphPad Prism version 6.

3. Results

3.1. ERβ antibody validation

Given the confusion in the ERβ field and the concern associated with variable and potentially non-specific reagents, we sought to extensively validate commonly used ERβ antibodies in a systematic manner that does not rely upon *a priori* assumptions regarding ERβ expression in cell line models or in tissues. As a control, we employed a cell line system with doxycycline-inducible expression of the ERβ protein, allowing us to assess antibodies in ERβ negative and matched ERβ positive conditions (Fig. 1A). One hundred-fold induction of ERβ mRNA in MDA-MB-231-ERβ cells treated with doxycycline 0.1 μg/ml for 24 h ($p = 0.01$) was confirmed by qRT-PCR (Fig. 1B).

Western blots of MDA-MB-231-ERβ+ and MDA-MB-231-ERβ− cell lysates with 8 different ERβ antibodies were performed (Fig. 1C). Six commonly used antibodies in the literature were included; PPG5/10 (Asgari and Morakabati, 2011; Carder et al., 2005; Ciucci et al., 2014; Shaaban et al., 2003; Wimberly et al., 2014), NCL-ER-BETA (Ellem et al., 2014; Hussain et al., 2012; McPherson et al., 2007; 2010; Morais-Santos et al., 2015; Oliveira et al., 2007; Umekita et al., 2006; Yang et al., 2015; Zellweger et al., 2013), Genetex 70182 (Celhay et al., 2010; Madak-Erdogan et al., 2013; Mak et al., 2013, Mak *et al.*, 2015, ; Nakajima et al., 2011), Millipore 06-629 (Bouchal et al., 2011; Chen et al., 2009; Grubisha et al., 2012), Abcam 288 [14C8] (Abd Elmageed et al., 2013; Carder et al., 2005; Colciago et al., 2014; Cotrim et al., 2013; Dey et al., 2012, 2014; Setlur et al., 2008; Shaaban et al., 2003; Vivar et al., 2010; Yang et al., 2012) and Santa Cruz 8974 (Al-Bader et al., 2011; Foryst-Ludwig et al., 2008; Han et al., 2015; Rossi et al., 2011; Zhou et al., 2012) antibodies. The PPG5/10 antibody detected a protein band of 77 kDa with no difference between

ERβ+ or ERβ− conditions, suggesting it is recognizing a non-specific protein. Similarly, the NCL-ER-BETA antibody detected a band of ~59 kDa, which is the correct size for ERβ however, there was no difference between ERβ+ or ERβ− conditions implying that this band was not ERβ. The GeneTex 70182 antibody detected a band of 59 kDa with differential signal between ERβ+ and ERβ− conditions, and a non-specific band was present at around 65 kDa. The Millipore 06-629 antibody detected a band of 59 kDa in both ERβ+ and ERβ− conditions, however the band was stronger in the ERβ+ condition, suggesting that the antibody could be cross-reacting with another protein of 59 kDa in addition to detecting ERβ. MC10, CWK-F12, Abcam 288 [14C8] and sc8974 ERβ antibodies all detected protein bands of 59 kDa with differential signal between ERβ+ and ERβ− conditions, confirming their specificity for ERβ by Western blotting. Further confirmation of the specificity of CWK-F12 to ERβ was demonstrated by IHC of MDA-MB-231-ERβ+ and MDA-MB-231-ERβ− cell pellets (Fig. 2), showing differential nuclear staining between the two conditions. The 8 ERβ antibodies were then assessed by an independent method called RIME, which uses an antibody-based purification followed by mass spectrometry (MS) to identify enriched peptides. We conducted RIME in MDA-MB-231-ERβ− and MDA-MB-231-ERβ+ cells using all 8 antibodies. E2F1 antibody was included in parallel as a positive control since E2F1 is a ubiquitous protein (Fig. 3A) and an IgG was used as a negative control (Fig. 3C). In MDA-MB-231-ERβ− cells, no ERβ peptides were purified by any of the ERβ antibodies, confirming the ERβ negative status of the uninduced MDA-MB-231-ERβ cell line (Fig. 3C). Following ERβ induction, RIME revealed diverse coverage of the ERβ protein by the different antibodies. The percent coverage of the ERβ protein following purification with each of the ERβ antibodies, and the location of the peptide fragments identified by MS are shown in Fig. 3B. To provide an indication of the specificity of each antibody, we ranked all the proteins purified by the IP and identified by MS according to the number of unique peptides (confirmed with a false discovery rate (FDR) of <1%). We hypothesized that the higher the ranking of ERβ, the greater the specificity of the antibody. Hence, if ERβ has the greatest number of unique peptides relative to all other proteins, it is ranked 1st.

NCL-ER-BETA did not purify any ERβ peptides (Fig. 3B), which is consistent with the lack of specificity identified from the Western blot result (Fig. 1C). The Millipore 06-629 antibody positively pulled down ERβ in the test condition, although coverage and ranking were not as favorable as compared with some of the other antibodies. Interestingly, LACTB, a 60 kDa protein was purified by Millipore 06-629 in both ERβ+ and ERβ− conditions (data not shown), which may explain the ~60 kDa band identified from Western blotting. Whilst the PPG5/10 did not detect ERβ by Western blotting, by RIME it detected ERβ with 25% coverage, with ERβ ranking 3rd in the list of identified peptides, suggesting differences in the specificity of this antibody from one experimental assay to another. PPG5/10 has been previously validated for IHC in a doxycycline-inducible U2OS-ERβ cell line, developed using the same plasmids as the MDA-MB-231-ERβ cell line (Wu et al., 2012). The Abcam 288 [14C8] antibody is a very commonly used ERβ antibody (Abd Elmageed et al., 2013; Colciago et al., 2014; Cotrim et al., 2013; Dey et al., 2012, 2014; Setlur et al., 2008; Shaaban et al., 2003; Vivar et al., 2010; Yang et al., 2012), which performed well by Western blotting, and also had the best antibody coverage by RIME (31.9%). However ERβ ranked 20th in the list of identified peptides when using Abcam 288 [14C8], suggesting that this antibody might also be purifying additional non-specific proteins. The MC10 antibody had the second-greatest coverage (28.2%) with ERβ ranking 1st in the list of identified peptides. In view of this finding, along with the positive Western blot result (Fig. 1), the MC10 antibody was carried forward into the RIME experiments for the cell line characterization. The CWK-F12 antibody had 17.7% coverage, with ERβ ranking 2nd in the list of purified peptides. As the CWK-F12 antibody produced very clean results by Western blotting, IHC and ranked ERβ second in the list of purified proteins, it was used for Western blotting in the cell line characterization and directly compared against the non-specific NCL-ER-BETA antibody. The goal was to use independent validated ERβ antibodies and additional independent methods to assess whether the most commonly studied breast and prostate cancer cell line models express ERβ.

3.2. Characterization of LNCaP and MCF-7 cell lines for ERβ expression

Given the wealth of publications assessing ERβ in breast (MCF-7) and prostate (LNCaP) cancer cell lines (Abd Elmageed et al., 2013; Al-Bader et al., 2011; Bouchal et al., 2011; Chen et al., 2009; Dey et al., 2014; Ellem et al., 2014; Fuqua et al., 1999; Hinsche et al., 2015; Kim et al., 2002; Lau et al., 2000; Mak et al., 2013; Shaaban et al., 2003; Skliris et al., 2002; Weng et al., 2013; Yang et al., 2012, 2015; Zhou et al., 2012), we sought to investigate the expression of ERβ in these models, using the newly validated ERβ antibodies. Protein lysate and RNA was collected from LNCaP and MCF-7 cells. Using primers validated in the inducible MDA-MB-231-ERβ cell line, which binds to sequence common to wild type (wt) ERβ and its isoforms (Fig. 1B), LNCaP and MCF-7 were shown to express no detectable levels of ERβ mRNA (Fig. 4A). Using the validated CWK-F12 ERβ antibody, ERβ protein was undetectable by Western blotting in these cells. By way of contrast, using the NCL-ER-BETA antibody on the same cell lysates, we detected a protein band of approximately 59 kDa in all conditions tested, including the MDA-MB-231-ERβ− cell line, confirming the non-specificity of this antibody to ERβ (Fig. 4B). Importantly, this demonstrates that the NCL-ER-BETA antibody is not detecting ERβ in either LNCaP or MCF-7 cancer cell line models and is instead identifying a non-specific protein of similar molecular weight.

Furthermore, RIME analysis of LNCaP and MCF-7 cells using the validated MC10 ERβ antibody did not purify any ERβ peptides by MS (Fig. 4C). This result was confirmed by an antibody-independent approach known as Parallel Reaction Monitoring (PRM), which demonstrated that no ERβ peptides were present in either of these cell lines (Fig. 4D). As such, our early passage LNCaP and MCF-7 cell line models are ERβ negative and these cancer models should not be used for the analysis of this protein.

3.3. ERβ expression in prostate and breast tissue

Importantly, whilst the LNCaP and MCF-7 cell-line models do not express ERβ, application of the validated CWK-F12 ERβ antibody to prostate and breast cancer TMAs demonstrated variable ERβ expression in differing cancer grades. In prostate tissue, previous reports have described an inverse correlation between ERβ expression and increasing Gleason grade of prostate cancer (Asgari and Morakabati, 2011; Attia and Ederveen, 2012; Dey et al., 2014; Horvath et al., 2001; Leav et al., 2001; Risbridger et al., 2007), whereas others have reported an association between increased ERβ expression and higher Gleason grade (Zellweger et al., 2013) or increased expression of ERβ in bone and lymph node metastases (Bouchal et al., 2011; Zhu et al., 2004). In our prostate TMA (Fig. 5A–D) we observed high expression of ERβ in the basal epithelium of benign glands, with no expression in Gleason grade 3 cancer. Gleason grade 4 cancer showed weak nuclear staining of ERβ and in areas of Gleason grade 5 cancer, ERβ nuclear expression was of moderate intensity. In breast tissue, previous studies have shown greatest ERβ expression in benign tissue, with a gradual

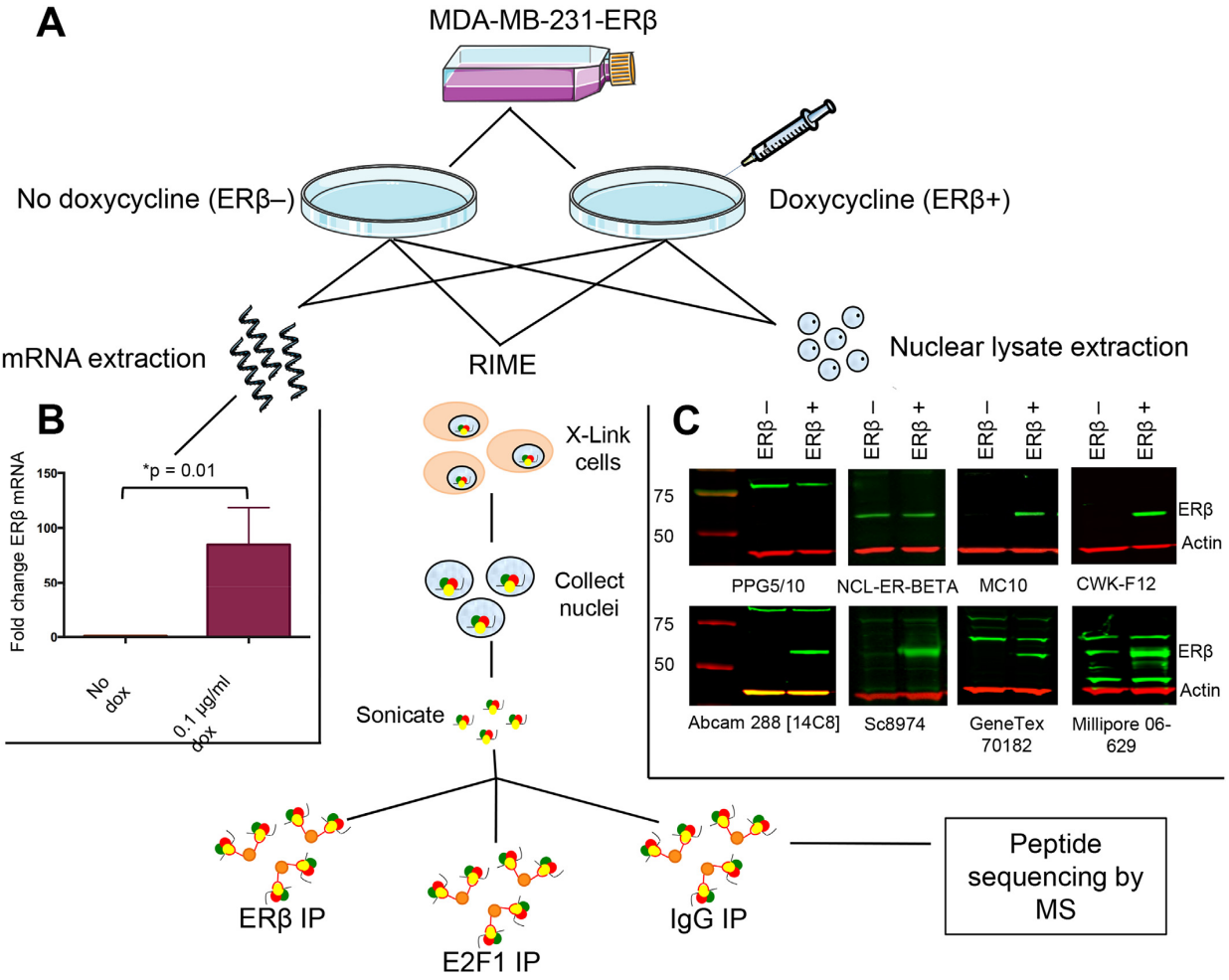

Fig. 1. Validation of ERβ antibodies using doxycycline-inducible MDA-MB-231-ERβ cells. (A) MDA-MB-231-ERβ cells were treated with doxycycline to induce ERβ expression. Untreated cells provided an ERβ-negative control. Messenger RNA was extracted for qRT-PCR and protein for Western blotting. MDA-MB-231-ERβ+ and MDA-MB-231-ERβ− cells were crosslinked and immunoprecipitated with antibody for RIME. (B) qRT-PCR confirmed 100-fold induction of ERβ mRNA in MDA-MB-231-ERβ+ cells versus untreated MDA-MB-231-ERβ− cells. Data are mean ± S.D. of technical triplicate experiments. (C) Western blots of MDA-MB-231-ERβ+ and MDA-MB-231-ERβ− cells with the 8 antibodies undergoing assessment. The MC10, CWK-F12, Abcam 288[14C8] and sc8974 antibodies detected bands of 59 kDa, with differential signal in the ERβ+ versus ERβ− conditions, indicating specificity to ERβ. GeneTex 70182 detected ERβ, although there was non-specific signal at 65 kDa. Millipore 06-629 appears to detect ERβ, although there is also a 59 kDa band in the ERβ− condition. Review of the RIME data suggests this may be cross-reactivity with LACTB. NCL-ER-BETA, the most commonly used ERβ antibody, gives bands of the correct size for ERβ, but there is no difference between ERβ− and ERβ+ conditions, confirming that this antibody is not specific to ERβ.

decrease in expression associated with increasing cancer grade (Guo et al., 2014; Omoto et al., 2002). Conversely, a non-statistically significant trend towards higher ERβ expression in Grade 3 tumors has also been reported (Myers et al., 2004). In our breast TMA (Fig. 5F–I), we observed greatest expression of ERβ in benign epithelium, with a trend towards decreasing ERβ expression associated with increasing cancer grade.

One potential explanation for the inconsistencies in ERβ tissue expression is the presence of ERβ splice-variant isoforms, which are fully conserved in exons 1–6, but have differing C-terminal domains (Leung et al., 2006). Different antibodies that bind either to the conserved regions or only to the C-terminal domain of the full-length ERβ protein may therefore give differing results (Supplementary Fig. 1). This may particularly be the case in prostate cancer, where it has been reported that ERβ isoform expression increases with the development of CRPC (Dey et al., 2012; Leung et al., 2010). Whilst this is likely to have an impact, our data suggest that some of the differing conclusions around ERβ expression in primary tissues are a direct result of certain investigations utilizing non-specific reagents that lack specificity for ERβ.

4. Discussion

Despite a large body of published literature, the role of ERβ in cancers of the prostate and breast is not clear. Contradictions between IHC findings and antibody-dependent molecular biology methods have contributed to this uncertainty, particularly the lack of clear consensus regarding correlation between tissue expression of ERβ and clinico-pathological parameters (Asgari and Morakabati, 2011; Attia and Ederveen, 2012; Bouchal et al., 2011; Dey et al., 2014; Esslimani-Sahla et al., 2004; Guo et al., 2014; Hieken et al., 2015; Horvath et al., 2001; Leav et al., 2001; Leygue and Murphy, 2013; Myers et al., 2004; Omoto et al., 2002; Risbridger et al., 2007;

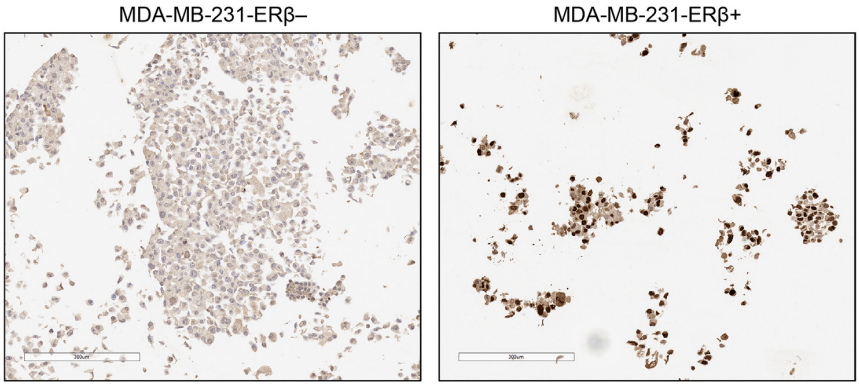

Fig. 2. IHC validation of CWK-F12 ERβ antibody in MDA-MB-231-ERβ cell pellets. Nuclear staining is evident in MDA-MB-231-ERβ+ cells and absent from the MDA-MB-231-ERβ– control, confirming the specificity of CWK-F12 to ERβ.

Roger et al., 2001; Umekita et al., 2006; Zellweger et al., 2013; Zhu et al., 2004).

Our results have demonstrated marked variation in the ability of commonly used commercially available ERβ antibodies to accurately detect ERβ by Western blotting and protein purification-MS based methods. Most notably, NCL-ER-BETA, a commonly used antibody (Ellem et al., 2014; Hussain et al., 2012; McPherson et al., 2007; 2010; Morais-Santos et al., 2015; Oliveira et al., 2007; Umekita et al., 2006; Yang et al., 2015; Zellweger et al., 2013) did not detect ERβ by any methodological approach. This antibody consistently yields bands on Western blots of the appropriate size for ERβ (59 kDa) in all tested conditions (Figs. 1C and 3B), but we have confirmed that this protein band is not ERβ through the use of the MDA-MB-231-ERβ inducible cell line system and the RIME technique. As such, this non-specific ~59 kDa band is likely to be the source of much of the controversy and confusion surrounding the study and characterization of ERβ. The PPG5/10 antibody targets the C-terminus of wt ERβ, and as such may be useful for distinguishing wt ERβ from expression of ERβ isoforms. PPG5/10 identified ERβ in the MDA-MB-231-ERβ cell line by RIME, and has previously been shown to be ERβ-specific by IHC in both an inducible cell line model (Wu et al., 2012) and in breast tissue (Carder et al., 2005). However, in our study this antibody did not show specificity by Western blot analysis (Fig. 1C). In their antibody validation study, Carder et al. also assessed the Abcam 288[14C8] antibody and found it to be ERβ-specific for IHC in tissue (Carder et al., 2005). Whilst our Western blotting data support this assertion (Fig. 1C), our RIME data suggest that this antibody also purifies additional, non-specific peptides, and as such should be used with caution for IP-based methods (Fig. 3B). Taken together, these findings reassert the importance of validating antibodies for individual experimental approaches, rather than assuming general applicability across methodological platforms (Baker, 2015; Bordeaux et al., 2010).

RIME was initially developed as a discovery tool to study the interacting proteomes of transcription factors in an unbiased manner (Mohammed et al., 2013). The advantage of using RIME in antibody validation arises from being able to identify specific, named peptides purified by an antibody, rather than relying on the presence of a protein band of approximate size on a Western blot. This is typified by the NCL-ER-BETA antibody, which gave bands on Western blot in both ERβ– and ERβ+ conditions and no ERβ peptides identified by RIME. Taken together, these data confirm that this antibody is not specific to ERβ. The non-commercially available ERβ antibodies tested (MC10 and CWK-F12) have been previously validated by other approaches (Choi et al., 2001; Wu et al., 2012) and our results add further confidence in their specificity using multiple independent assays. By comparing the peptide coverage of each antibody along with the ERβ ranking (as a surrogate of specificity) RIME facilitated an informed decision-making process in selecting which antibody to carry forward to the cell-line characterization. Our multi-modal approach to cell-line characterization using both antibody-dependent (Western blotting and RIME) and antibody-independent (qRT-PCR and PRM) approaches has shown that low-passage, genotyped LNCaP and MCF-7 cell lines do not express detectable ERβ, despite numerous publications making conclusions about ERβ biology using these cell line models (Abd Elmageed et al., 2013; Al-Bader et al., 2011; Bouchal et al., 2011; Chen et al., 2009; Dey et al., 2014; Ellem et al., 2014; Fuqua et al., 1999; Hinsche et al., 2015; Kim et al., 2002; Lau et al., 2000; Mak et al., 2013; Weng et al., 2013; Yang et al., 2012, 2015). Whilst we acknowledge that immortalized cell lines may have variable expression of certain factors across passage numbers and laboratories (Masters, 2000), our data suggest the need for caution in making this assumption with respect to ERβ. Reassuringly, we have confirmed expression of ERβ in prostate and breast tissue using the validated CWK-F12 antibody. Our IHC study is not intended to be an exhaustive analysis of ERβ expression in prostate and breast tissue, and we acknowledge the limitations presented by our small sample size and lack of statistical correlation with clinico-pathological parameters. We have however, demonstrated that the CWK-F12 ERβ antibody is validated for IHC and in principle can be used for larger scale assessment of ERβ expression in tissue.

Epidemiological evidence suggests that estrogen and its receptors have important roles in the development and progression of prostate cancer. Japanese men are known to have a very low incidence of prostate cancer (Ross et al., 1992), and it has been proposed that their traditional diet, which is high in ERβ selective phytoestrogens may exert a protective role (Andres et al., 2011; Attia and Ederveen, 2012; Hori et al., 2011; Messina, 2010; Reiter et al., 2011; Shen et al., 2000; Stettner et al., 2007; Thelen et al., 2007, Thelen et al., 2005; Wuttke et al., 2002). Further evidence from studies of ERβ knockout mice (βERKO) shows a clear phenotype and tumor-suppressive effect of ERβ (Ricke et al., 2008). However, clinical trials of agents seemingly effective *in vitro* have demonstrated no clinical benefit of estrogen-selective agents in prostate cancer (Bergan et al., 1999; Kim et al., 2002). There are numerous explanations as to why this might be, for example, expression of ERβ in non-epithelial cell types (Gargett et al., 2002; Pierdominici et al., 2010) modulating the tissue response to these

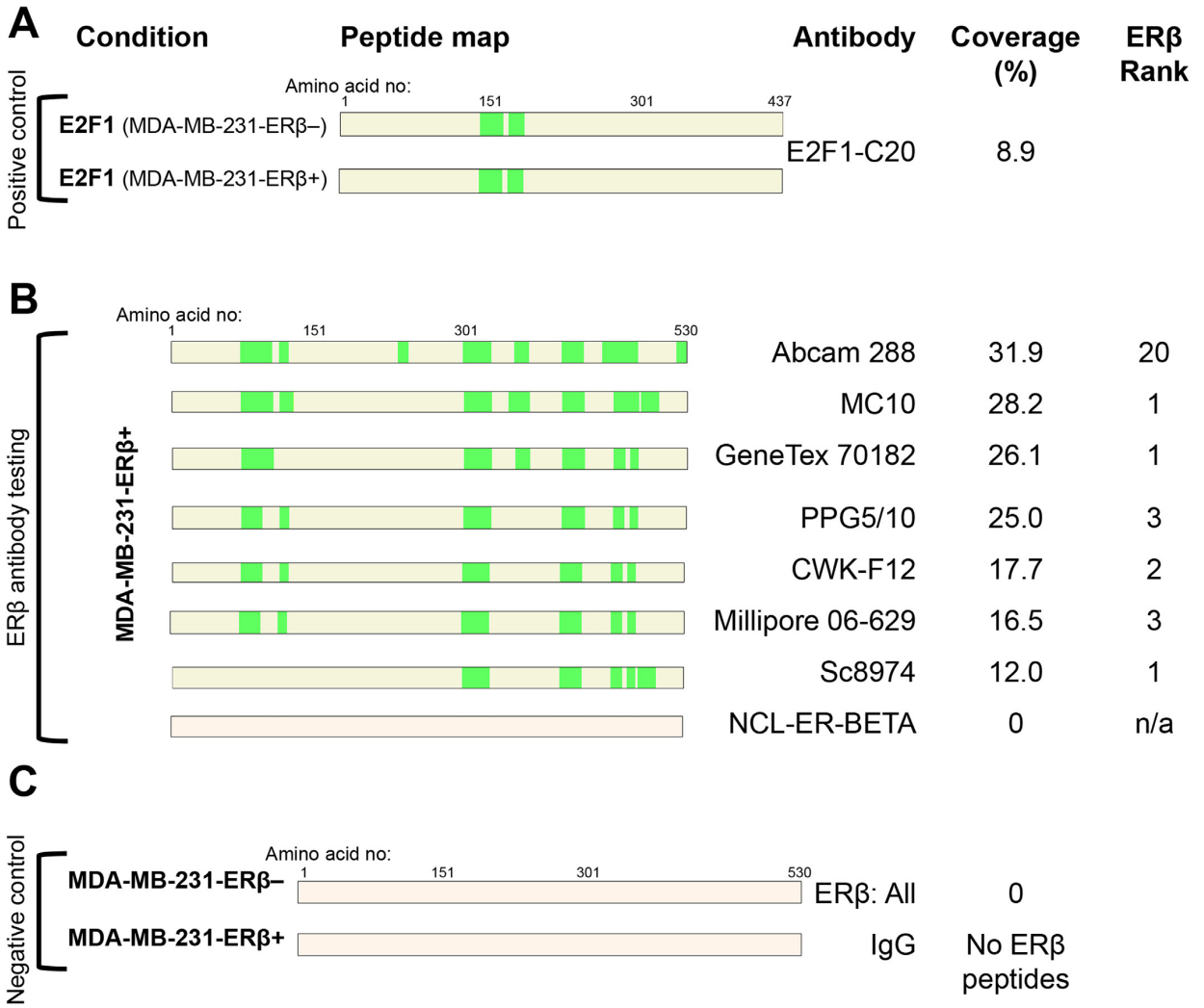

Fig. 3. RIME demonstrates specificity and peptide coverage of ERβ antibodies. Eight ERβ antibodies were assessed by RIME in MDA-MB-231-ERβ+/– cells. Coverage of the protein relates to green areas on the peptide maps, indicating peptides identified by MS with false discovery rate of ≤1% (mean of 2 biological replicates). (A) E2F1 antibody was applied to MDA-MB-231-ERβ– and MDA-MB-231-ERβ+ conditions as a positive control, as E2F1 is a ubiquitously expressed protein. (B) ERβ antibody tests: 'ERβ ranking' indicates where ERβ features in a list of proteins purified by the antibody, ranked by number of unique peptides identified in MS, giving an indication of antibody specificity. NCL-ER-BETA failed to identify ERβ. (C) Negative controls: All of the ERβ antibodies were tested in MDA-MB-231-ERβ– cells, to confirm absence of ERβ expression in the non-induced condition. Mouse IgG antibodies were used to identify non-specific peptides pulled down by the IP. None of the IgG antibodies purified ERβ.

agents, but in light of our findings we would suggest that use of poorly validated reagents and inadequately characterized cancer cell line models is an important contributing factor.

In the presented study, detailed validation of commonly used ERβ antibodies has demonstrated that some of these reagents either detect ERβ in specific experimental conditions only or lack any specificity for ERβ across multiple assays. ERβ has been investigated in numerous cancers including prostate, breast, kidney (Yu et al., 2013), colon (Dey et al., 2013), endometrium (Han et al., 2015), ovary (Ciucci et al., 2014; Suzuki et al., 2008) bladder (Hsu et al., 2013) and non-small cell lung cancer (He et al., 2015; Luo et al., 2015) but in many cases, the findings are predicated on non-specific reagents. As such, a re-evaluation of ERβ expression and biology is needed using reliable, specific reagents. Our determination of ERβ antibody specificity will contribute towards clarifying existing, conflicting data on the role of ERβ in these diverse cancers and provide the necessary, validated tools with which to move forward our understanding of ERβ biology.

Ethics

Prostate tissue included in the tissue microarray was obtained under approval granted for the ProMPT study (MREC/01/4/061). Breast tissue collection and assessment was approved by the University of Adelaide Human Research Ethics Committee (#s H-2005-065).

Financial support

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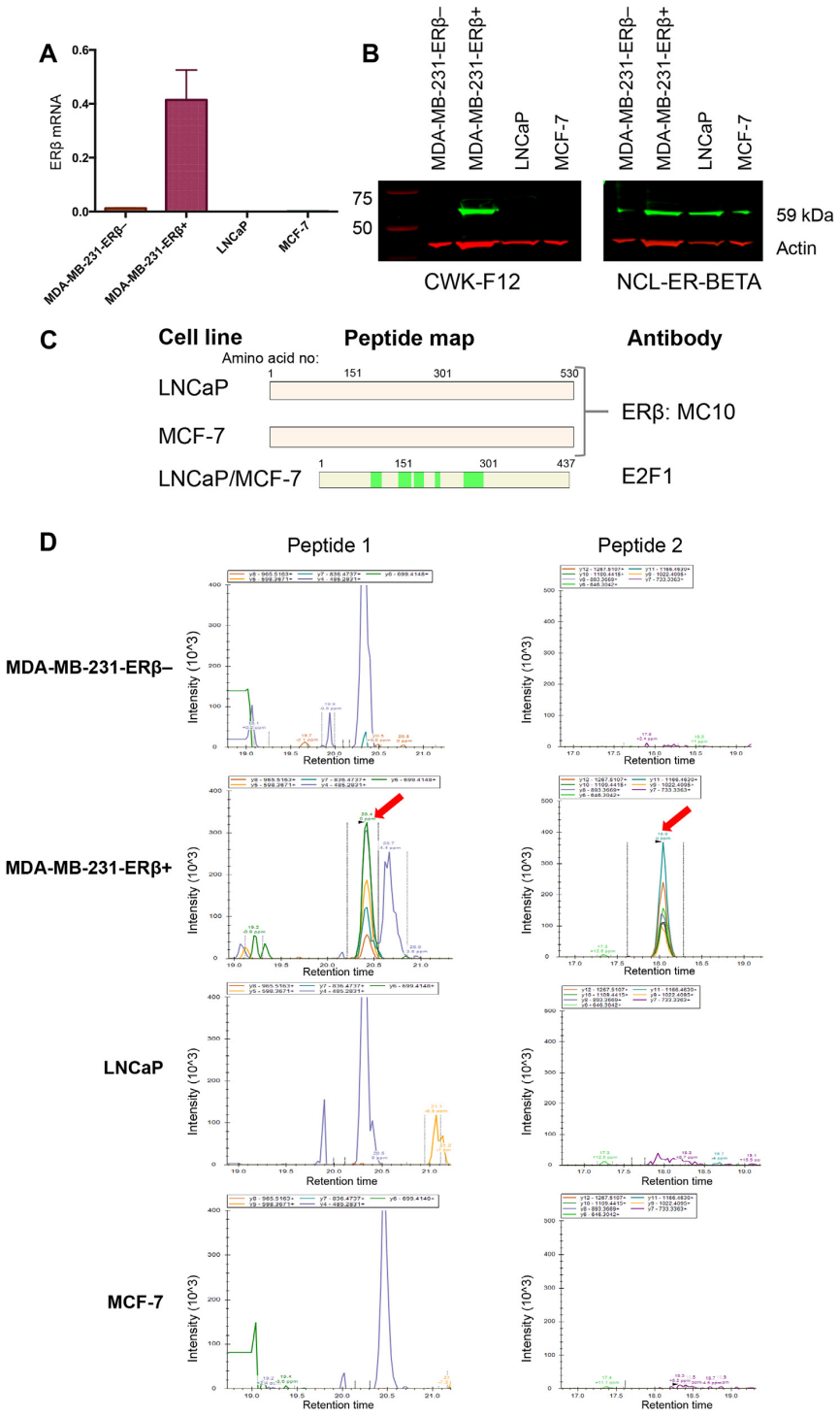

Fig. 4. Multimodal characterization of LNCaP and MCF-7 cell lines confirms absence of ERβ expression. LNCaP and MCF-7 are cell lines commonly used to study ERβ. We detected no ERβ expression in either cell line at mRNA level by qRT-PCR (A) or at protein level by western blot (B) or RIME (C) using validated CWK-F12 and MC10 antibodies respectively. Western blot of the same cell lysates using the NCL-ER-BETA antibody clearly shows how some of the conflicting data in the literature has arisen, as this antibody shows bands of the correct size for ERβ in all conditions including MDA-MB-231-ERβ– negative control. (D) PRM confirms, using an antibody-independent technique, the absence of ERβ protein expression in LNCaP and MCF-7 cells. The ERβ peptides (peptide 1 is SLEHTLPVNR and peptide 2 is SSITGSECSPAEDSK) identified in the MDA-MB-231-ERβ+ positive control (red arrows) are absent in the other cell lines. Data shown are representative of 2 independent biological replicates.

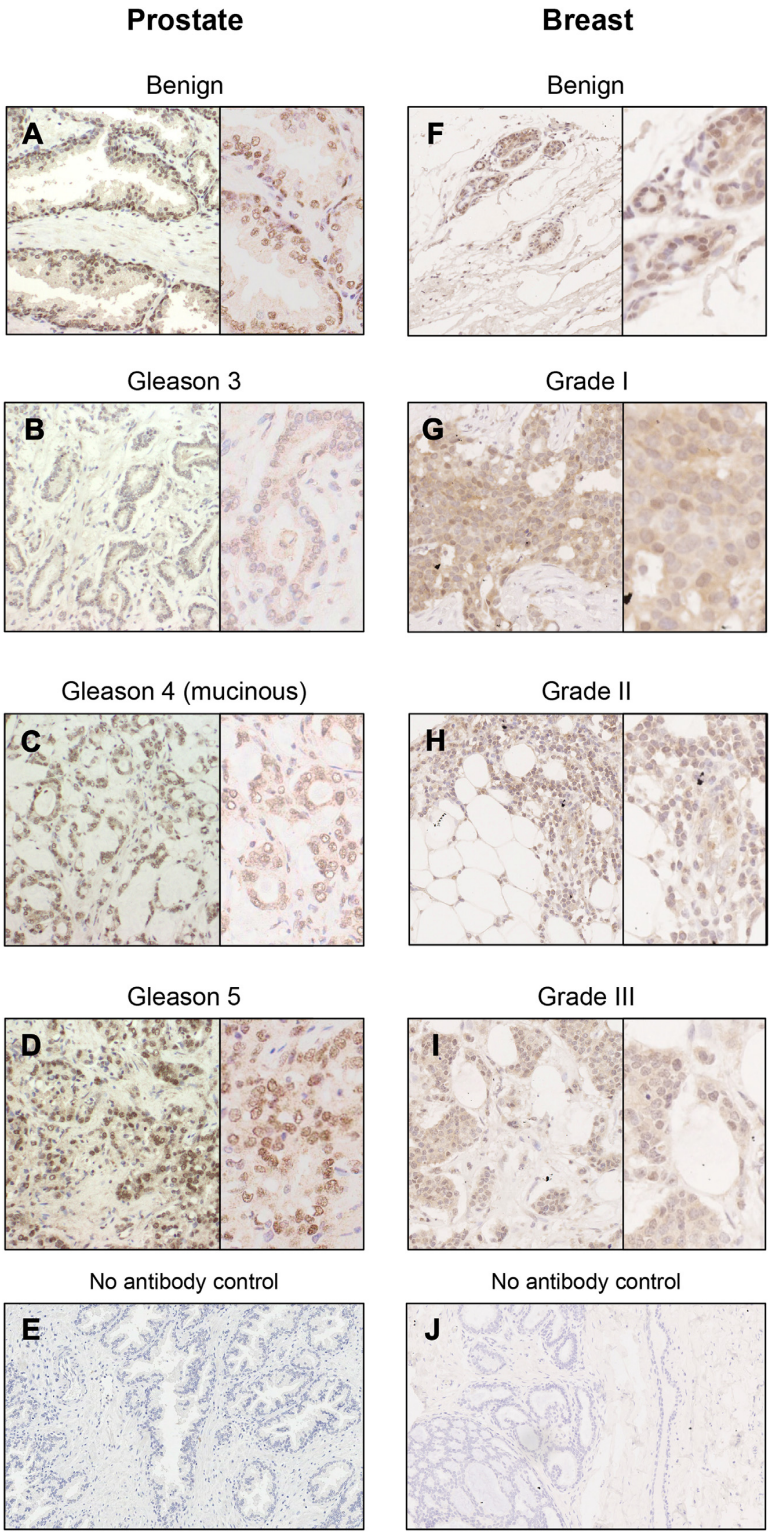

Fig. 5. IHC of prostate and breast tissue with validated CWK-F12 ERβ antibody. Demonstration of variable ERβ expression in differing grades of prostate (A–D) and breast (F–I) cancer. In prostate, ERβ was highly expressed in basal and luminal epithelial cells of benign glands (A), whereas there was no nuclear staining in Gleason grade 3 cancer (B). In Grade 4 mucinous tumor (C) and high grade tumor (D) nuclei showed weak to moderate expression of ERβ. In breast, ERβ expression was greatest in nuclei of benign epithelial cells (F), which was observed to decrease with increasing grade of cancer (G, H and I – Grade 1, 2 and 3 respectively). The greatest difference in expression was observed between benign (F) and Grade 3 cancer (I). E and I - no primary antibody negative controls.

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Conflicts of interest

The authors confirm there are no conflicts of interest to disclose.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.mce.2016.11.016.

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Appendix B

List of abbreviations

1. **ADT**; Androgen deprivation therapy
2. **AF**; Activation function
3. **AR**; Androgen receptor
4. **ARE**; Androgen response element
5. **ArKO**; Aromatase knock-out
6. **aERKO**; ERα knock-out
7. **bERKO**; ERβ knock-out
8. **BCR**; Biochemical relapse
9. **ChIP**; Chromatin immunoprecipitation
10. **COX2**; Cyclo-oxygenase 2
11. **CRPC**; Castrate resistant prostate cancer
12. **CSS**; Cancer specific survival
13. **CT**; Computed tomography
14. **DBD**; DNA-binding domain
15. **DES**; Diethylstilbestrol

16. **DHEA**; Dihydroepiandrosterone
17. **DHT**; Dihydrotestosterone
18. **EBRT**; External beam radiotherapy
19. **ER**α; Estrogen receptor alpha
20. **ER**β; Estrogen receptor beta
21. **ERE**; Estrogen response element
22. **EMT**; Epithelial-mesenchymal transition
23. **FDR**; False discovery rate
24. **GR**; Glucocorticoid receptor
25. **HN**; hormone naïve
26. **HPG**; Hypothalamo-pituitary-gonadal
27. **IHC**; Immunohistochemistry
28. **IMRT**; Intensity modulated radiotherapy
29. **IP**; Immunoprecipitation
30. **LBD**; Ligand-binding domain
31. **LHRH**; Leutinising hormone releasing hormone
32. **MR**; Mineralocorticolid receptor
33. **MRI**; Magnetic resonance imaging
34. **MS**; Mass spectrometry
35. **NO**; Nitric oxide
36. **NTD**; N-terminal domain
37. **PC**; Prostate cancer

38. **PIN**; Prostatic intraepithelial neoplasia
39. **PR**; Progesterone receptor
40. **PRM**; Parallel reaction monitoring
41. **PSA**; Prostate specific antigen
42. **RIME**; Rapid immunoprecipication and mass spectrometry of endogenous protein
43. **RP**; Radical prostatectomy
44. **SERM**; Selective estrogen receptor modulator
45. **STAT**; Signal transducer and activator of transcription
46. **Tet-R**; Tetracycline repressor
47. **TMA**; Tissue microarray
48. **TNM**; Tumour, nodes, metastasis

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